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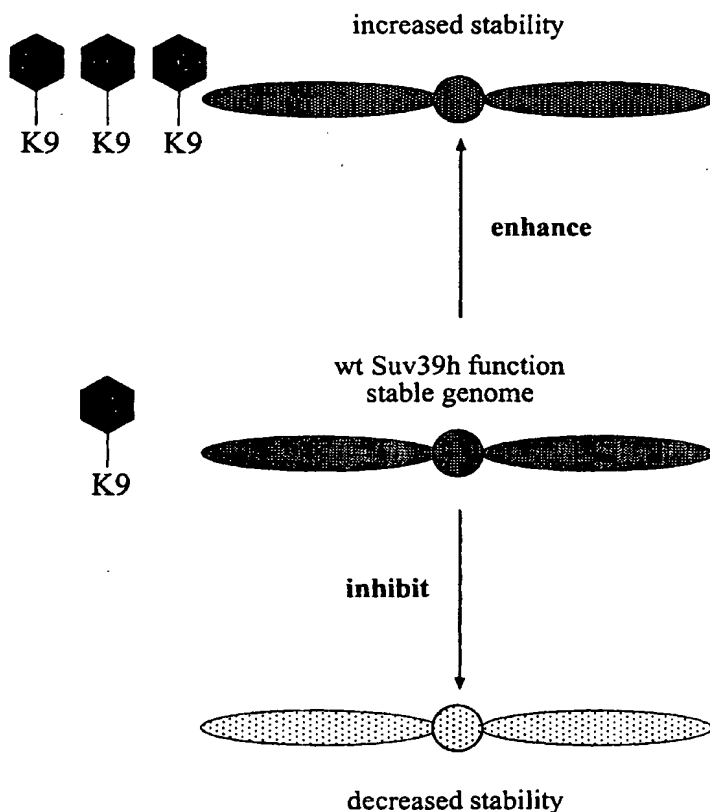
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(54) Title: SCREENING-METHOD FOR MODULATORS OF METHYLTRANSFERASE-DEPENDENT CHROMOSOME STABILITY



(57) Abstract: A method for identifying compounds that alter higher order chromatin dependent chromosome stability is based on determining the compounds' ability to modify a methyltransferase with Suv39h-like methyltransferase activity. The identified compounds are useful in therapy, in particular the therapy of human cancer and for contraception.

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SCREENING-METHOD FOR MODULATORS OF METHYLTRANSFERASE-DEPENDENT CHROMOSOME STABILITY

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The invention relates to a method for identifying compounds influencing chromosome dynamics in eukaryotic cells. In particular, the invention relates to the treatment and prevention of human conditions by modulating higher order chromatin dependent chromosome stability during mitosis and
10 meiosis.

Higher-order chromatin is essential for epigenetic gene control and for the functional organisation of chromosomes. Differences in higher-order chromatin structure have been linked with distinct covalent modifications of histone tails which regulate transcriptional 'on' or 'off' states (Grunstein,
15 1998; Turner, 1998; Strahl and Allis, 2000) and influence chromosome condensation and segregation (Karpen and Allshire, 1997; Wei *et al.*, 1999).

Histones constitute a highly conserved family of proteins (H3, H4, H2A, H2B, H1) which are the major components of eukaryotic chromatin
20 structure. Histones compact genomic DNA into basic repeating structural units, the nucleosomes. In addition to their DNA packaging function, histones have been proven to be integral components of the molecular machinery that regulates gene expression.

Post-translational modifications of histone N-termini, particularly of H4 and
25 H3, are well documented and have functionally been characterised as

changes in acetylation (Grunstein, 1998; Turner, 1998; Strahl and Allis, 2000), phosphorylation (Wei *et al.*, 1999) and, most recently, methylation (Chen *et al.*, 1999; Strahl *et al.*, 1999). In contrast to the large number of described histone acetyltransferases (HATs) and histone deacetylases (HDACs), genes encoding enzymatic activities that regulate phosphorylation (Sassone-Corsi *et al.*, 1999; Hsu *et al.*, 2000) or methylation (Chen *et al.*, 1999) of histone N-termini are only beginning to be identified. Moreover, the interdependence of the different histone tail modifications for the integration of transcriptional output or higher-order chromatin organisation is currently not understood.

Overall, there is increasing evidence that the regulation of normal and aberrant cellular proliferation is not only affected on the transcriptional level, but that also a higher level of regulation is involved, i.e. the organisation of chromatin structure through the modification of histone molecules. The determination of the proteins and the molecular mechanisms involved in histone modification will contribute to the understanding of the cellular proliferation program and will thus shed light on the mechanisms involved in aberrant proliferation occurring in tumour formation and progression (Jacobson and Pillus, 1999).

Genetic screens for suppressors of position effect variegation (PEV) in *Drosophila* (Reuter and Spierer, 1992) and *S.pombe* (Allshire *et al.*, 1995) have identified a subfamily of approximately 30-40 loci which are referred to as *Su(var)*-group (Wallrath, 1998) genes. Interestingly, several histone deacetylases (De Rubertis *et al.*, 1996), protein phosphatase type 1 (Baksa *et al.*, 1993) and S-adenosyl methionine synthetase (Larsson *et al.*, 1996) have been classified as *Su(var)*s. In contrast, *Su(var)2-5* (which is allelic to *HP1*) (Eissenberg *et al.*, 1992), *Su(var)3-7* (Cléard *et al.*, 1997) and *Su(var)3-9* (Tschiersch *et al.*, 1994; Schotta and Reuter, 2000) encode heterochromatin-associated proteins. *Su(var)* gene function thus suggests

a model, in which modifications at the nucleosomal level may initiate the formation of defined chromosomal subdomains that are then stabilised and propagated by heterochromatic SU(VAR) proteins (Henikoff, 1997).

Su(var)3-9 is dominant over most PEV modifier mutations (Tschiersch *et al.*, 1994), and mutants in the corresponding *S.pombe clr4* gene (Ivanova *et al.*, 1998) disrupt heterochromatin association of other modifying factors and result in chromosome segregation defects (Ekwall *et al.*, 1996). Recently, human (*SUV39H1*) and murine (*Suv39h1* and *Suv39h2*) *Su(var)3-9* homologues have been isolated (Aagaard *et al.*, 1999). It has been shown that they encode heterochromatic proteins which associate with mammalian HP1 (Aagaard *et al.*, 1999). The SU(VAR)3-9 protein family combines two of the most evolutionarily conserved domains of 'chromatin regulators': the chromo (Aasland and Stewart, 1995; Koonin *et al.*, 1995) and the SET (Tschiersch *et al.*, 1994; Jenuwein *et al.*, 1998) domain. Whereas the 60 amino acids chromo domain represents an ancient histone-like fold (Ball *et al.*, 1997) that directs eu- or heterochromatic localisations (Platero *et al.*, 1995), the molecular role of the 130 amino acids SET domain has remained enigmatic. Overexpression studies with human SUV39H1 mutants indicated a dominant interference with higher-order chromatin organisation that, surprisingly, suggested a functional relationship between the SET domain and the distribution of phosphorylated (at serine 10) histone H3 (Melcher *et al.*, 2000).

It was an object of the invention to gain further insight into the molecular pathways leading to histone modifications and higher-order chromatin organisation in order to harness these findings for interfering with aberrant gene expression and genomic instability through chromosome mis-segregation and thus provide new cancer therapies.

In particular, it was an object of the invention to investigate the function of members of the SU(VAR)3-9 protein family with the view to develop novel

strategies to affect higher-order chromatin dependent chromosome stability. Such strategies can be employed in therapies for the treatment of conditions in which aberrant gene expression and genomic instability through chromosome mis-segregation are causally involved. (The term
5 "chromosome stability" implies successful segregation of chromosomes resulting in the maintenance of a stable karyotype).

Examples 1 to 7 of the present invention show that mammalian SU(VAR)3-9 related proteins (human SUV39H1, murine Suv39h1 and murine Suv39h2) are SET domain-dependent H3-specific histone
10 methyltransferases which selectively methylate lysine 9 ("K9") of the H3 N-terminus. Methylation of K9 negatively regulates phosphorylation of adjacent serine 10 and reveals a 'histone code' that appears intrinsically linked to the organisation of higher-order chromatin. (In the following, histone methyltransferases are termed "HMTases" or, more generally,
15 "MTases").

After having identified Suv39h1 and Suv39h2 as mammalian histone H3 lysine 9 specific histone methyltransferases (Suv39h HMTases), it was shown that these HMTases are heterochromatin-enriched enzymes which transiently accumulate at centromeres during mitosis (Aagaard *et al.*, 1999;
20 Aagaard *et al.*, 2000). Moreover, it was shown that methylation of histone H3 at lysine 9 (H3-K9) creates a high-affinity binding site for HP1 proteins (Lachner *et al.*, 2001; Bannister *et al.*, 2001), thereby defining the SUV39H1-HP1 methylation system as a crucial regulatory mechanism for the assembly and propagation of heterochromatin (Jenuwein, 2001).
25 Overexpression of human SUV39H1 induces ectopic heterochromatin and results in chromosome mis-segregation in mammalian cell lines (Melcher *et al.*, 2000). In addition to the essential mitotic functions described above, heterochromatin is also crucial for the dynamic reorganization of meiotic chromosomes. Meiosis is initiated by chromosomal movements from the

nuclear lumen to the nuclear envelope, where chromosomes cluster via their pericentric satellite sequences (Hawley *et al.*, 1992; Scherthan *et al.*, 1996). At meiotic prophase, chromosomes condense, followed by homolog pairing and recombination (at pachytene) between maternal and paternal
5 chromosomes. The onset of the meiotic divisions is preceded by desynapsis, further chromosome condensation and histone H3 phosphorylation at pericentric heterochromatin (Cobb *et al.*, 1999). In particular for male germ cells, the haploid genome content is finally organized into one heterochromatic block in elongating spermatids. In
10 *Drosophila*, heterochromatin and its associated satellite sequences have been proposed to assist in the initial meiotic chromosome movements and in homolog pairing by orienting chromosomes along a similar higher-order structure (Hawley *et al.*, 1992; Karpen *et al.*, 1996; Dernburg *et al.*, 1996b). In germ cells of mammals, a pachytene checkpoint (de Vries *et al.*, 1999)
15 monitors mis-aligned and unpaired chromosomes and arrests cells in meiotic prophase, thereby preventing the production of aneuploid gametes.

It was a further object of the invention to analyse the role of Suv39h1 and Suv39h2 in embryonic development and in spermatogenesis in view of utilizing these proteins as drug targets for conditions involving fertility, in
20 particular male fertility.

To solve the problems underlying the present invention, in a first step bioinformatics techniques were applied. Using the SET domains of the SU(VAR)3-9 protein family as a starting alignment, significant sequence and secondary structure similarities (see Methods) to six plant protein
25 methyltransferases were detected.

To investigate whether the SET domain of human SUV39H1 has enzymatic activity, histones were tested as possible substrates for *in vitro* methylation. The obtained results demonstrate that SUV39H1 harbors an intrinsic

histone methyltransferase activity and suggest that this HMTase activity resides in the C-terminal SET domain.

Using recombinant proteins, both murine GST-Suv39h1(82-412) and the corresponding human SUV39H1 fusion protein [GST-SUV39H1(82-412)]
5 were shown to be catalytically active. Short internal deletions were introduced into the two conserved regions of the SET domain core in GST-SUV39H1(82-412), and additional mutants lacking the C-terminal tail (Δ C-tail) or the SET-associated cysteine-rich region (Δ cys) were generated. All mutant proteins failed to demonstrate HMTase activity.

10 Although these results suggest a significant contribution by the cysteine-rich regions, their apparent absence in the plant methyltransferases does not prevent catalytic activity. To investigate enzyme function of the SET domain in more detail, point mutations were introduced into the most highly conserved motif. *In vitro* HMTase assays indicated that all point mutations,
15 with the exception of one, abolished enzymatic activity. Surprisingly, the latter mutation resulted in an hyperactive enzyme with approximately 20-fold increased activity. The data obtained define the 320 H ϕ NHSC326 motif in the SET domain as an important catalytic site.

Because the SET domain is one of the most conserved protein motifs in
20 chromatin regulators (Stassen *et al.*, 1995; Jenuwein *et al.*, 1998), it was next analysed whether SU(VAR)3-9 family members or other SET domain proteins contain HMTase activity. GST-fusion products of the extended SET domains of *S.pombe* CLR4 (Ivanova *et al.*, 1998), human EZH2 (Laible *et al.*, 1997) and human HRX (Tkachuk *et al.*, 1992) were generated
25 that would correspond to GST-SUV39H1(82-412). Interestingly, GST-CLR4(127-490) displayed pronounced HMTase activity at three- to five-fold increased levels as compared to the recombinant SUV39H1 product, consistent with CLR4 carrying an arginine at the hyperactive position. The results obtained from this analysis show, in agreement with

the mutational analysis of SUV39H1, that HMTase activity towards free histones appears to require the combination of the SET domain with adjacent cysteine-rich regions, which is a quality found in only a restricted number of SET domain containing proteins.

5 These experiments indicated that the HMTase activity of mammalian SU(VAR)3-9 related proteins is selective for histone H3 under the chosen assay conditions. To examine this finding in more detail, *in vitro* methylation reactions were performed with individual histones. It could be shown that H3 is specifically methylated by GST-Suv39h1(82-412), whereas no signals
10 are detected with H2A, H2B or H4. Methylation of H3 has been shown to occur predominantly at lysine 4 in a wide range of organisms, as well as at lysine 9 in HeLa cells, although the responsible HMTase(s) have yet to be defined (Strahl *et al.*, 1999). To investigate the site utilisation profile of Suv39h1, unmodified peptides comprising the wild-type H3 N-terminus and
15 a mutant K9L peptide were tested as substrates. Additionally, insulin and peptides comprising the N-termini of CENP-A (Sullivan *et al.*, 1994), macroH2A (Pehrson and Fried, 1992) were included. These *in vitro* assays revealed selective methylation of the wild-type H3 peptide. The data obtained also suggested that the H3 N-terminus is a preferred residue for
20 Suv39h1-dependent HMTase activity.

To more definitively determine this site preference, the wild-type H3 N-terminal peptide was *in vitro* methylated by GST-Suv39h1(82-412), using S-adenosyl-[methyl-³H]-L-methionine. The labelled peptide, purified by reverse-phase HPLC, was then directly microsequenced, and
25 ³H-incorporation associated with each individual amino acid was analysed. The results confirmed selective transfer of methyl-label to lysine 9, demonstrating that Suv39h1 is a highly site-specific HMTase for the H3 N-terminus *in vitro*.

- Murine *Suv39h* genes are encoded by 2 loci, *Suv39h1* and *Suv39h2*. To investigate the *in vivo* significance of *Suv39h* function and *Suv39h* dependent K9 H3 methylation, mouse strains deficient for both *Suv39h1* and *Suv39h2* were generated. *Suv39h1* and *Suv39h2* deficient strains were intercrossed to produce *Suv39h* double deficient mice. Double mutant mice were born in sub-Mendelian ratios. Some double null embryos exhibited severe growth retardation and exencephaly. In addition surviving double mutants were growth retarded, suggesting a role for *Suv39h* in cell proliferation.
- 10 In order to determine whether the embryonic phenotypes in *Suv39h* null mice can be attributed to mitotic defects, PMEFs (primary mouse embryonic fibroblasts) derived from *Suv39h* double mice were analysed. *Suv39h* double null PMEFs display a reduced G1-index and an increased proportion of cells with aberrant nuclear morphologies, reminiscent of
- 15 division defects during mitosis. Furthermore, double null cells also show genomic instabilities and readily become aneuploid. The severity of these aneuploidies increases with higher passage numbers. The inability of *Suv39h* double null cells to maintain a stable karyotype may underlie the *Suv39h* embryonic phenotype.
- 20 Phosphorylation at serine 10 (phosH3) in the N-terminal tail of H3 has been shown to be required for condensation and subsequent segregation of chromosomes (Wei *et al.*, 1999). During the cell cycle, phosH3 initiates within pericentric heterochromatin in late G2 and then progresses along the entire chromosomes during mitosis (Hendzel *et al.*, 1997). It was found that
- 25 in wild-type PMEFs, approximately 7% of the cells stain positive for the characteristic, heterochromatin-associated phosH3 foci. In contrast, this number is increased by a factor of about 3-fold in *Suv39h* double null PMEFs. This result suggested that the overall levels of phosH3 may be enhanced in *Suv39h* double null PMEFs. This was confirmed

biochemically. Together, the obtained data are most consistent with a model in which Suv39h-mediated methylation of lysine 9 in H3 negatively regulates phosphorylation of serine 10.

Together, these data clearly demonstrate crucial roles for *Suv39h* during
5 cell division. Loss of *Suv39h* function impairs K9 histone H3 metylation and induces defective cell division resulting in genome instabilities. Segregation defects/genome instability underlies the aetiology of many human cancers (Lengauer *et al.*, 1997) and are often a prerequisite for tumour progression. These observations make *Suv39h* an excellent candidate for novel
10 therapeutic approaches for tumour therapies.

In additon, a set of experiments of the present invention provides *in vivo* evidence that the absence of Suv39h HMTase activities impairs development and viability of mutant mice, and directly correlates with a nearly complete lack of H3-K9 methylation at pericentric heterochromatin.
15 Notably, Suv39h-deficient mice display chromosomal instabilities in both somatic and meiotic cells that are further evidenced by an increased risk for development of B-cell lymphomas and perturbed chromosome interactions during male meiosis. These *in vivo* data assign a fundamental role for H3-K9 methylation at pericentric heterochromatin and suggest that the Suv39h
20 HMTases regulate a 'heterochromatic competence' which protects chromosome stability during mitosis and meiosis.

Single gene disruptions for either *Suv39h1* or *Suv39h2* allow for normal mouse development and do not appear to affect viability and fertility of mutant mice. This apparent redundancy in gene function would be
25 consistent with the overlapping expression profile of the two *Suv39h* genes during mouse embryogenesis (O'Carroll *et al.*, 2000). By contrast, combined disruption of both genes in *Suv39h* double null (dn) mice results in severely impaired perinatal viability ($\approx 33\%$; Table I), growth retardation and hypogonadism in males. Both *Suv39h* dn males and are infertile.

Although *Suv39h* dn fetuses appear to develop normally until day E12.5, they then display smaller body sizes and frequently are resorbed during late gestation. These *in vivo* analyses indicate an important role(s) for the *Suv39h* genes during mammalian development and for overall viability.

5 Since the absence of Suv39h HMTase activities induces genomic instabilities, the high lethality of *Suv39h* dn fetuses could mainly be a consequence of perturbed chromosome segregation which would significantly impair the proliferation and differentiation programmes of the developing embryo.

10 Although Suv39h enzymes are the major HMTases for H3-K9 methylation at pericentric heterochromatin in somatic cells (see Figure 12) and in early meiotic cells (see Figure 13B), there are ≤ 15 unique gene sequences in the mouse genome that contain the evolutionarily highly conserved SET domain and which are likely to encode additional enzymes with putative
15 HMTase activity (Jenuwein, 2001). At least one of these SET domain containing proteins can indeed also methylate H3 at lysine Tachibana *et al.*, 2001 Thus, the $\approx 33\%$ viability of *Suv39h* dn mice could be dependent on the compensating activity of other HMTases that may be expressed to varying degrees in the mixed genetic background, in which the *Suv39h* dn
20 mice have been analyzed.

Absence of Suv39h HMTase activities triggers genomic instabilities in a variety of cell types, including mouse embryonic fibroblasts (PMEFs) (see Figure 10), fetal liver and bone marrow cells and in spermatogonia (see Figure 14C). In agreement with the aneuploidies observed in these cellular
25 systems, *Suv39h*-deficient mice display an increased risk for tumorigenesis, resulting in late-onset B-cell lymphomas in $\approx 33\%$ of *Suv39h* dn mice (see Figure 11). B-cell lymphomas also develop upon reduced *Suv39h* gene dosage in compound mutant mice that contain gene disruptions of *Suv39h1* (see Table II). Intriguingly, the *Suv39h*-induced

aneuploidies are mainly characterized by segregation failure of the nearly complete set of the chromosomes, resulting in hypo-tetraploid or even hypo-octaploid cells (see Figure 10). These data suggest a general impairment of chromosome segregation, consistent with the lack of H3-K9
5 methylation around all acrocentric centromeres in *Suv39h* dn cells (see Figure 12).

Distinct modifications of histone N-termini, such as acetylation (Ekwall *et al.*, 1997) and phosphorylation (Wei *et al.*, 1999) have been shown to be required for correct chromosome segregation in *S.pombe* and
10 *Tetrahymena*, presumably by inducing a specialised chromatin structure at pericentric heterochromatin that facilitates the establishment of a functional centromere. Because H3-K9 methylation restricts H3 phosphorylation mediated by the Ipl1/aurora kinase (Hsu *et al.*, 2000) and is also interdependent with histone acetylation (Rea *et al.*, 2000), the absence of
15 *Suv39h* HMTase activities is likely to perturb this distinct histone modification pattern. Second, in addition to altering nucleosome arrangements, histone modifications can generate specific interaction affinities for chromatin-associated proteins (Rice and Allis, 2001). Although the localisation of CENP epitopes appears unaltered, heterochromatic
20 enrichment of HP1 proteins is largely lost in *Suv39h* dn somatic cells (Lachner *et al.*, 2001). Notably, HP1 interacts *in vitro* with INCENP (Ainsztein *et al.*, 1998) which forms a complex with aurora-B (Adams *et al.*, 2000; Kaitna *et al.*, 2000). Mutation of INCENP induces severe mitotic abnormalities including macronuclei and internuclear bridges, and results in
25 nearly complete chromosome mis-segregation and cytokinesis failure (Cutts *et al.*, 1999; Adams *et al.*, 2000; Kaitna *et al.*, 2000). These intriguing parallels suggest a possible *in vivo* link between *Suv39h*-mediated H3-K9 methylation and aurora-B dependent phosphorylation, and could categorize *Suv39h* genes as novel tumour suppressor genes.

The *Suv39h*-mediated chromosomal instabilities only affect a sub-population of cells and do not appear to trigger pronounced apoptosis (see Figure 10b), consistent with similar analyses of *clr4* mutants in *S.pombe* (Ekwall *et al.*, 1996; Ivanova *et al.*, 1998). These data suggest that the

5 *Suv39h*-induced defects in somatic cells are not under strict surveillance of known checkpoint controls (Cortez and Elledge, 2000; but see Bernard *et al.*, 1998) and may be caused by rather late segregation problems during mitosis. Indeed, a fraction of *Suv39h* dn cells contain chromosomes that lag at anaphase. Since *Suv39h* dn PMEFs are characterized by hypo-

10 tetraploid and hypo-octaploid karyotypes (see Figure 10d) and tumor cells contain 'butterfly' chromosomes (see Figure 11c), a model is proposed (Figure 16, top panel), in which the absence of H3-K9 methylation would allow stronger or more persistent pericentric associations between aligned metaphase chromosomes. Although a role for the *Suv39h* HMTases in

15 centromeric cohesion remains to be determined, it provides an attractive mechanism to explain possible cytokinesis failure and mis-segregation of the entire chromosome complement without activating known checkpoint controls.

In contrast to somatic cells, *Suv39h*-mediated defects in male meiosis

20 induce pronounced apoptosis of stage V - VI spermatocytes during the transition from mid to late pachytene (see Figure 13a). Activation of programmed cell death at this stage has also been observed in mouse mutants that are impaired in DNA damage control (Xu *et al.*, 1996), meiotic recombination (Yoshida *et al.*, 1998; de Vries *et al.*, 1999; Baudat *et al.*,

25 2000) and synaptonemal complex formation (Yuan *et al.*, 2000). In *Suv39h* dn spermatocytes, pericentric H3-K9 methylation is specifically reduced at the pre-leptotene stage but, surprisingly, appears as a wild-type staining during later meiotic stages (see Figure 13b, bottom panel). Thus, in analogy to the increased centromeric associations discussed above, it is

30 proposed that impairment of H3-K9 methylation at the onset of meiosis

induces aberrant centromere clustering that can no longer be 'rescued' by the hypothetical activity of additional H3-K9 HMTases during mid-pachytene. This model (see Figure 16, bottom panel) would characterize Suv39h-dependent H3-K9 methylation as one of the earliest requirements to ensure successful meiosis and to prevent illegitimate heterochromatic interactions. Because non-homologous interactions will result in delayed synapsis or even complete pairing failure (see Figure 14a), they trigger apoptosis by activating the pachytene checkpoint (de Vries *et al.*, 1999), thereby protecting the male germ line from accumulating aneuploidies.

10 In *Suv39h* dn mice, spermatogenic failure is promoted by illegitimate chromosomal interactions, synaptic delay, unpaired sex chromosomes and bivalent mis-segregation at meiosis I (see Figures 14 -15). Notably, a major fraction of these 'forbidden' interactions comprises physical contacts between the sex chromosomes and autosomes that are largely mediated through centromeric regions (see Figure 14D and 14J). These data suggest that the impairment of H3-K9 methylation may allow pericentric heterochromatin to form a more relaxed configuration which is prone to become engaged in random associations. Cytological and genetic studies in *Drosophila* demonstrated the intrinsic potential of heterochromatin to restrict inter- and intrachromosomal interactions (Dernbrug *et al.*, 1996a; Csink and Henikoff, 1996). Moreover, pericentric heterochromatin has been shown to initiate and maintain alignment and pairing of achiasmate chromosomes until meiosis I (Karpen *et al.*, 1996; Dernburg *et al.*, 1996b), suggesting a role for heterochromatin in defining a 'self-complementary' higher-order chromosome structure that would ensure partner recognition of homologous chromosomes (Karpen *et al.*, 1996). The *in vivo* data on the function of the Suv39h HMTases would be consistent with these proposed roles of heterochromatin and reveal the first evidence that impaired definition of meiotic heterochromatin can affect chromosome identity in a mammalian organism.

Finally, *Suv39h* deficiency induces uni-valency of the sex chromosomes at pachytene and at diakinesis (see Figure 15). Intriguingly, HP1 β (Motzkus *et al.*, 1999; Turner *et al.*, 2000) and the Suv39h2 HMTase (O'Carroll *et al.*, 2000) localise to the specialised chromatin structure of the sex
5 chromosomes in the XY body. Although XY body formation appears normal in early/mid pachytene of *Suv39h* dn spermatocytes, *Suv39h* deficiency prolongs H3-K9 methylation (see arrows in Figure 13B) and induces hypocondensation of the Y chromosome (see Figure 15E). These results involve the Suv39h HMTase activities in the definition of the heterochromatic
10 identity of the Y chromosome and suggest that *Suv39h*-mediated H3-K9 methylation may indirectly promote or stabilise homolog pairing of the heteromorphic sex chromosomes.

Heterochromatin has been first described more than 70 years ago (Heitz, 1928). Because of its stable appearance in the cell nucleus, it has been
15 proposed to serve crucial functions for the inheritance of cell type identities and the fidelity of chromosome segregation. The discoveries of the first HMTases (Rea *et al.*, 2000; O'Carroll *et al.*, 2000) and their mechanistic link to generate a heterochromatic affinity through H3-K9 methylation and recruitment of HP1 proteins (Lachner *et al.*, 2001; Bannister *et al.*, 2001;
20 Nakayama *et al.*, 2001) has now defined an entry point to start dissecting some of the basic roles of heterochromatin.

The experiments of the present invention have provided *in vivo* evidence that H3-K9 methylation at pericentric heterochromatin is indeed a crucial requirement to ensure mammalian development and to protect
25 chromosome stability in both somatic cells and male germ cells. Because *Suv39h* deficiency impairs chromosome function in mitosis and meiosis, the data assign a fundamental role for H3-K9 methylation in directing a 'heterochromatic competence' for overall chromosome dynamics and

identity - and reveal some of the direct biological functions of the enigmatic entity called heterochromatin.

In a first aspect, the results obtained in the experiments of the present invention show that members of the SU(VAR)3-9 protein family have
5 HMTase activity which identifies them as novel targets for the therapy of proliferative disorders, in particular cancer.

Furthermore, the experiments of the invention demonstrate that the Suv39h HMTases are important for embryonic development and spermatogenesis.

Combined disruption of both *Suv39h* HMTase genes abolishes H3-K9
10 methylation at pericentric heterochromatin and induces chromosomal instabilities with an increased risk for tumorigenesis.

In addition, *Suv39h* double null male mice display complete spermatogenic failure that is largely caused by non-homologous chromosome associations and delayed synapsis, resulting in apoptosis of meiotic prophase cells.
15 Together, these results establish histone H3-K9 methylation as a crucial determinant for pericentric heterochromatin and provide a direct role for the Suv39h HMTases in maintaining a 'heterochromatic competence' that protects chromosome stability during mitosis and meiosis.

The identification of members of the SU(VAR)3-9 protein family,
20 exemplified by human SUV39H1, murine Suv39h1 and murine Suv39h2, as K9 specific histone H3 MTases is the prerequisite for designing assay methods that allow for finding compounds altering, in particular interfering with, higher order chromatin dependent chromosome stability, which is the basis for novel approaches in cancer therapy. (In the following, if not
25 otherwise stated, the term "Suv39h" refers to both the murine and the human protein).

Due to the role of Suv39h1 or Suv39h2 in spermatogenesis, compounds

modulating the MTase activity of these proteins and thus modulating spermatogenesis may also be used in the treatment of male infertility (using compounds that enhance Suv39h MTase activity) and for reversible male contraception (using compounds that inhibit Suv39h MTase activity).

- 5 The present invention relates to a method for identifying a compound that alters higher order chromatin dependent chromosome stability during mitosis and meiosis, said method comprising incubating a substrate for a methyltransferase, in the presence of a methyl donor, with a MTase with Suv39h-like MTase activity, in the presence or absence of a test compound
10 and determining whether the compound modulates the MTase activity.

The group of MTases with Suv39h-like MTase activity (in the following also termed "Suv39h-like MTases") encompasses enzymes which display histone H3 K9 MTase activity or methyltransferase activity for other yet to be identified substrates.

- 15 The term "histone H3 K9" is not limited to the human SUV39H or mouse Suv39h substrate (i.e. the methylation site of histone H3 at lysine 9), but is meant to encompass any substrate of the histone or histone variant-type of protein, the methylation of which results in the below-defined epigenetic signal.

- 20 Additional members of the group of MTases can be identified by bioinformatic/biochemical techniques and tested biochemically. By way of example, in a first step, by searching data bases for similarities, as described in Example 1. Next, an identified candidate can be verified as a MTase with Suv39h-like MTase activity in biochemical assays similar to or
25 identical with those described in the Examples.

This group of Suv39h-like MTases also encompasses MTases with specificities for other histone H3 residues than K9 or for substrates other than histone H3, which are, like the Suv39h K9 histone H3 HMTase activity

observed in the present invention, required for higher order chromatin dependent chromosome stability. This epigenetic signal may be a consequence of histone methylation at lysine 9 on H3 alone; however, it cannot be excluded that MTase activity on undefined substrates or a
5 combination of substrate methylation and other covalent modifications, such as phosphorylation or acetylation, at other histone residues are involved.

In the experiments of the present invention, Suv39h variants with point mutations in the SET domain were shown to confer hyperactive HMTase
10 activity to the protein, these Suv39h variants may be advantageously used in the method of the invention.

In a preferred embodiment, the MTase is mouse Suv39h1 or Suv39h2, most preferably, the MTase is human SUV39H1 or SUV39H2.

Since it has been shown in the present invention that recombinant Suv39h
15 retains HMTase activity, most preferably, a recombinant MTase is employed. Suv39h or Suv39h variants can be produced recombinantly according to standard methods by expression in suitable hosts, e.g. bacteria, yeast, insect or eukaryotic cells and purified, e.g. on glutathione-agarose columns if it has been tagged with GST.

20 The Suv39h1 and SUV39H1 cDNA sequences are known from the literature (Aagaard *et al.*, 1999), the Suv39h2 cDNA sequence is shown in SEQ ID NO: 1; the human SUV39H2 cDNA is defined by the ESTs as shown in SEQ ID NO: 3 - 6.

In the case of testing the compounds for their effect on Suv39h activity, the
25 assay comprises, as its essential features, incubating a histone H3 protein or a histone H3 N-terminal fragment including K9, a methyl donor, e.g. methionine or S-adenosyl-L-methionine, with a preparation containing a

Suv39h MTase activity and determining MTase activity in the presence or absence of a test substance.

MTase substrates useful in the method of the invention may be those equivalent to or mimicking the naturally occurring substrates, e.g.

5 biochemically purified histone H3, recombinantly produced histone H3, or a histone H3 peptide that contains the K9 methylation site, or other yet to be identified proteins which act as substrates for Suv39h MTases. Novel Suv39h substrates can be identified by bioinformatic and biochemical techniques and tested using the biochemical assays described in the
10 Examples of the present invention. For example, novel Suv39h substrates can be identified by co-immunoprecipitation techniques. Suv39h proteins or tagged versions of Suv39h proteins can be immunoprecipitated with specific antisera and interacting proteins identified by mass spectroscopy techniques. A yeast two hybrid screen using Suv39h proteins or portions of
15 Suv39h proteins as a bait can also be employed to identify novel interacting protein from a variety of cDNA libraries.

In a preferred embodiment, the histone H3 fragment ARTKQTARKSTGGKAPRKQL (SEQ ID NO:7) is employed. Alternatively, a modified peptide may be used for which the MTase has increased
20 affinity/activity. Such peptides can be designed by exchanging and/or adding and/or deleting amino acids and testing the substrate in serial experiments for MTase affinity/activity.

The methyl group of the methyl donor preferably carries a detectable label, e.g. a radioactive or a chromogenic label, which can be quantified upon
25 transfer to the substrate.

Preferably, the methyl donor is radioactively labelled methionine or S-adenosyl-L-methionine.

Alternatively to using a labelled methyl donor, the substrate, upon methylation by the enzyme, is used to serve as an epitope which can be recognised by a specific antibody and hence be quantified by standard immunoassay techniques, e.g. ELISAs. Antibodies useful in this type of assay can be obtained by using the methylated substrate, preferably a small peptide, e.g. the peptide with the sequence shown in SEQ ID NO:7, as an antigen and obtaining polyclonal or monoclonal antibodies according to standard techniques. The generation and purification of a methyl-specific antibody against the histone H3 lysine 9 position is described in the Materials and Methods section. A suitable H3-K9 methyl antibody was also described by Nakayama *et al.*, 2001.

For small scale applications, the screening method can be based on an assay as described in Example 2, 3 or 4.

In an alternative embodiment, the screening method of the invention utilizes the fact that the methylation of histone H3 at lysine 9 (H3-K9) creates a high-affinity binding site for HP1 proteins. In this embodiment, the substrate, upon methylation, is allowed to bind to HP1 and then incubated with a labelled anti-HP1 antibody. The difference in label intensity between the reaction in the absence or presence of the test compound is indicative for the compound's modulating effect on MTase activity.

HP1 is preferably used in recombinant form. Based on the information of the HP1 cDNA sequence (Jones *et al.*, 2000; Accession No. BC006821), HP1 is produced recombinantly according to standard technology. The recombinant protein or fragments thereof are used to generate polyclonal or monoclonal antibodies that are employed in this assay format.

In a preferred embodiment, the method of the invention is performed on a high-throughput scale. For this embodiment, the major assay components, in particular Suv39h, are employed in recombinant form.

For the high throughput format, the screening methods of the invention to identify MTase inhibitors, are carried out according to standard assay procedures. Such assays are based on the catalytic transfer, mediated by Suv39h or a Suv39h variant, of a methyl group from a donor to a substrate, e.g. a histone H3 peptide. To achieve this, the substrate, e.g. histone H3 or a variant or fragment thereof, is immobilised on a carrier, usually a microtiter plate, and incubated with recombinant Suv39h or a Suv39h variant and a methyl donor.

The methyl group of the methyl donor carries a label, preferably a chromogenic or radioactive label.

Fluorescent or radioactive labels and the other reagents for carrying out the enzymatic reaction on a high-throughput scale are commercially available and can be employed according to the supplier's instructions (e.g. Molecular Probes, Wallac). Examples for suitable fluorescent labels are coumarin derivatives, e.g., 7-amino-4-methylcoumarin or 7-amino-4-trifluoromethylcoumarin. The radioactive label may be a ^{14}C or a ^3H atom. Upon transfer of the methyl group to the substrate by Suv39h, in the case of a chromogenic reagent, the methyl donor changes colour which can be quantified. In the case of using a radioactive methyl donor, the methyl group is transferred to the substrate and can be directly quantified.

The specific assay design depends on various parameters, e.g. on the size of the substrate used. In the the case of using a short peptide, the fluorescence quenching or the fluorescence resonance energy transfer methods are examples for suitable assay technologies, as described below.

The substrate may be tagged, e.g. with biotin, the reaction is then carried out in solution and then transferred to streptavidin coated microtiter plates, e.g. in the case of a radioactive methyl group, „flash“ plates, the material of

which contains the scintillant, or plates which are coated with scintillant. Thus the level of methylation of the substrate can be quantified in a suitable scintillation machine/reader. Alternatively, the assay can be carried out in the streptavidin coated „flash“ plates with the biotinylated substrate already bound to the plates. This type of assay may also be conducted in the form of a so-called „homogenous assay“ (an assay type which does not require intermediate transfer and washing steps) e.g. by using microbeads that are coated with scintillant and streptavidin, to which the biotinylated substrate is bound.

Similarly to biotin, other commonly used tags, e.g. Flag, Myc, HA, GST, that are suitable to immobilize the substrate to the plate that is coated with the tag-specific antibody, may be used in the above-described assays.

In a variant, this assay is conducted in the format ELISA type assay; in this case, a methyl-specific antibody is used to detect the amount of methylated substrate bound to the plate.

Alternatively, the plate is coated with an antibody against the methylated substrate to capture the methylated substrate; the substrate is also either tagged or chromogenically labeled and the amount of bound methylated tagged/labeled substrate can be quantified either by a tag-specific antibody or by measuring the level of chromogenic label. By way of example, the substrate is a linear or a branched peptide, e.g. [TARKST]₄-K₂-K-cys) that is labeled with a chromogenic label, e.g. europium, and upon methylation by a Suv39h-like MTase becomes an epitope for a Lys9-methyl specific antibody (see materials and methods) immobilised on a carrier (e.g. microtiter plate). The non-captured substrate is washed away, the europium label is then cleaved and its fluorescence enhanced and the level of fluorescence is calculated by time resolved fluorescence. The level of fluorescence is directly related to the level of methylated substrate (figure 17).

An alternative embodiment is based on the principle that methylation of the peptide may alter its sensitivity to cleavage by a protease. Utilizing this principle, the fluorescence quenching (Resonance Energy Transfer „RET“) assay may be employed to determine the amount of methylation of peptidic substrates. In a first step, a Suv39h peptidic substrate, which contains the methylation site and a recognition/cleavage site for a defined protease, that is sensitive to modification (in the particular case, methylation of the lysine) of the recognition/cleavage site, e.g. trypsin or LysC. The peptide carries a fluorescent donor near one end and an acceptor near the other end. In the uncleaved substrate, the fluorescence of the substrate is quenched by the persisting intramolecular RET between donor and acceptor. Upon cleavage of the (unmethylated) substrate by the protease, the cleavage products are released from RET quenching and a fluorescence signal is generated. Methylation of the substrate abolishes the ability of the protease to cleave the substrate. Thus, abolishment of the protease activity (which is proportional to methylation) is reflected by signal repression, in case of total protease inhibition, total signal repression to the basal level.

An assay of this type may be carried out as follows: the solution of the labeled substrate (e.g. the peptide labeled with 4-[[4'-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) at the one end and with 5-[(2'-aminoethyl)amino]naphthalenesulfonic acid (EDANS) at the other end or labeled with benzyloxycarbonyl at the one end and with 4-aminomethylcoumarin at the other end) in assay buffer is transferred into each well of black 96-well microtiter plates. After addition of the test substances in the defined concentration, the MTase and the methyl donor are added to the wells. After incubation under reaction conditions and for a period of time sufficient for the methylation reaction, e.g. for 40 min at room temperature, the protease, e.g. trypsin, is added and allowed to react under suitable conditions, finally, the fluorescence is measured in a fluorometer at

the excitation wavelength, e.g. at 340 nm, and at the emission wavelength, e.g. at 485 nm.

In the case of using the FRET assay, the following commercially available labeling pairs are suitable for the method of the invention: Europium (Eu) and Allophycocyanin (APC), Eu and Cy5, Eu and PE (Wallac, Turku, Finland). If a test substance is a modulator of the MTase activity, there will be, depending on the detection system and depending on whether the test substance has an inhibiting or an activating effect, a decrease or an increase in the detectable signal as compared to a control sample in the absence of a test substance. In the high-throughput format, compounds with a modulating effect Suv39h MTase activity can be identified by screening test substances from compound libraries according to known assay principles, e.g. in an automated system on microtiter plates.

By providing a method to identify compounds which exert their effect by directly modulating, in particular by inhibiting, a Suv39h-like MTase, the present invention provides a basis for inhibiting the proliferation of rapidly dividing animal cells, in particular tumour cells.

The compounds identified in the above methods, which are also subject of the invention, have the ability to interfere with chromosome stability and high fidelity chromosome segregation by modulating the MTase activity of Suv39h.

In a preferred embodiment, the compounds of the invention are inhibitors of Suv39h HMTase activity.

Preferably, the compounds are specific modulators of Suv39h, in particular Suv39h1 or Suv39h2.

The present invention also relates to compounds, which act as modulators of a Suv39h-like MTase activity, in particular modulators of Suv39h, for use in human therapy, in particular cancer therapy.

Compounds inhibiting Suv39h HMTase activity result in decreased genome
5 stability and can be used in therapy for targeting dividing cells, in particular highly proliferative tumour cells. They are preferably administered in combination with other genome destabilising agents, e.g. mitose inhibitors like tubulin binders (taxanes, e.g. taxol, Paclitaxel; or epithelones).

SUV39H inhibitors may also be used jointly with or before the application of
10 conventional tumour therapies, e.g. radiotherapy or chemotherapy, in particular DNA damaging agents, in order to pre-sensitize the tumour cells. By destabilizing the cell's genome, the SUV39H inhibitors make the cell more susceptible to the parallel/subsequent treatment.

The SUV39H inhibitors will preferably be used in a combination therapy
15 and applied in consecutive and transient treatments. Since the development of B-cell lymphomas in *Suv39h* double null mice only occurs with a late onset (i.e. after 9 months of age), transient treatments with SUV39H inhibitors should not induce an immediate increase in tumor risk but rather weaken overall genomic stabilities of highly proliferating cells.

20 Likewise, agents which enhance Suv39h HMTase activity can be used to stabilise the genome of inherently unstable cells, rendering them less prone to acquiring proliferation promoting mutations. A model for *Suv39h* function and effects of inhibition or enhancement of Suv39h enzymes is shown in Figure 8.

25 The efficacy of compounds identified as Suv39h modulators can be tested for *in vivo* efficacy in mammalian cells with *Suv39h* double null cells serving as a positive control. Compounds effective in cancer therapy should interfere with chromosome stability and segregation, which can be

measured by karyotyping, e.g. by analysing the DNA content by FACS or standard cytological techniques. Substances whose potential for therapeutic use has been confirmed in such secondary screens can be further tested for their effect on tumour cells. To test the inhibition of tumour cell proliferation, primary human tumour cells are incubated with the compound identified in the screen and the inhibition of tumour cell proliferation is tested by conventional methods, e.g. bromo-desoxy-uridine or ³H thymidine incorporation. Compounds that exhibit an anti-proliferative effect in these assays may be further tested in tumour animal models and used for the therapy of tumours.

Compounds intended for male fertility applications can be tested in animal models described by Vigil et al., 1985, in animal models developed for experimental studies of human spermatogenesis, as described by Weinbauer et al., 2001, or in animal models that mimic human male reproductive defects, as described by Lamb and Niederberger (1994). Guidance for a valid application of animal data to the assessment of human reproductive disorders is given by Working, 1988.

Toxicity and therapeutic efficacy of the compounds identified as drug candidates by the method of the invention can be determined by standard pharmaceutical procedures, which include conducting cell culture and animal experiments to determine the IC₅₀, LD₅₀, the ED₅₀. The data obtained are used for determining the human dose range, which will also depend on the dosage form (tablets, capsules, aerosol sprays, ampules, etc.) and the administration route (oral, buccal, nasal, paterental, rectal or, in the case of temporary male contraceptive applications, local sustained release form applications, e. g. slow-releasing micropellets that are implanted into or adjacent to the gonads). A pharmaceutical composition containing the compound as the active ingredient can be formulated in conventional manner using one or more physiologically active carriers and

excipients. Methods for making such formulations can be found in manuals, e.g. "Remington Pharmaceutical Sciences".

- As *Suv39h* is required to maintain a stable karyotype, it can be considered as a tumour suppressor gene. If *SUV39H* mutations also prove to be a
- 5 factor underlying cellular transformation events in humans, which is strongly indicated by the analysis of *Suv39h* double null mice in developing B-cell lymphomas, it can be expected that the re-introduction of a wild type *Suv39h* gene by gene therapy results in increased genomic stability delaying or inhibiting cancer progression.
- 10 For gene therapy, the *Suv39h* DNA molecules may be administered, preferably contained on a plasmid in recombinant form, directly or as part of a recombinant virus or bacterium. In principle, any method of gene therapy may be used for applying *Suv39h* recombinant DNA, both *in vivo* and *ex vivo*.
- 15 Examples of *in vivo* administration are the direct injection of "naked" DNA, either by intramuscular route or using gene guns. Examples of recombinant organisms are vaccinia virus or adenovirus. Moreover, synthetic carriers for nucleic acids such as cationic lipids, microspheres, micropellets or liposomes may be used for *in vivo* administration of nucleic acid molecules
- 20 coding for the *Suv39h* polypeptide.

Since it has been shown in the present invention that *Suv39h* mediates dynamic transitions in higher-order mammalian chromatin largely through its intrinsic HMTase activity, histone H3-K9 methylation (H3-K9 Me) represents an important epigenetic imprint for chromosome dynamics

25 during cell division. Hence, antibodies specific for H3-K9 Me can be used to screen cells/patients for heterochromatin based genome instabilities. In essence, H3-K9 methylation specific antibodies can be used as a diagnostic tool for human diseases associated with aberrant gene

expression and genomic instability through chromosome mis-segregation or with aberrant definition or organisation of heterochromatin.

Brief description of figures:

- 5 Figure 1: HMTase activity of transfected and recombinant SUV39H1 and Suv39h1 proteins.
- Figure 2: Specific HMTase activity of the SET domain of mammalian SU(VAR)3-9 related proteins.
- Figure 3: Histone H3 lysine 9 is the major site for *in vitro* methylation by recombinant Suv39h1.
- 10 Figure 4: Targeting of *Suv39h1* and *Suv39h2* in the mouse germline.
- Figure 5: Analysis of *Suv39h* double null PMEFs.
- Figure 6: Aberrant mitoses in *Suv39h* double null PMEFs.
- Figure 7: Increased phosH3 phosphorylation in *Suv39h* double null PMEFs.
- 15 Figure 8: Model for Suv39h HMTase function.
- Figure 9: Generation and genotyping of *Suv39h1*- and *Suv39h2*-deficient mice.
- Figure 10: Chromosomal instabilities in *Suv39h* dn PMEFs.
- Figure 11: Development of B-cell lymphomas in *Suv39h* mutant mice.
- 20 Figure 12: *Suv39h*-dependent H3-K9 methylation at pericentric heterochromatin.
- Figure 13: Spermatogenic failure and H3-K9 methylation in germ cells of *Suv39h* dn mice.

Figure 14: Illegitimate associations and delayed synapsis of *Suv39h* dn meiotic chromosomes.

Figure 15: Aberrant function of the Y chromosome during meiosis of *Suv39h* dn spermatocytes.

5 Figure 16: Model for a 'heterochromatic competence' in protecting chromosome stability.

Figure 17: Schematic illustration of a screening method for identifying *Suv 39h* modulators

10

Materials and Methods

a) Sequence alignments and secondary structure predictions

The SET domains of human SUV39H1 (Aagaard *et al.*, 1999), *Drosophila* SU(VAR)3-9 (Tschiersch *et al.*, 1994) and *S.pombe* CLR4 (Ivanova *et al.*,
15 1998) were used as a multiple starting alignment for database similarity searches using Profile (Birney *et al.*, 1996), hidden Markov (Eddy, 1998) and position-specific iterative BLAST (Altschul *et al.*, 1997) methods (representative listings are available from the SET domain page of the SMART WWW-server (Schultz *et al.*, 2000). These searches revealed
20 significant similarities to six plant proteins (accession numbers Q43088, O65218, P94026, O80013, AAC29137 and AC007576_12) described as putative lysine N-methyltransferases. For example, a PSI-BLAST search with the *S.pombe* hypothetical protein SPAC3c7.09 as query identified these plant sequences and well-known SET domain sequences within ten
25 rounds using an E-value inclusion threshold of 0.001. The same search also revealed the presence of a SET domain in YHR109w, which is known

to encode a cytochrome c MTase (Martzen *et al.*, 1999), within three rounds. Consensus secondary structures were predicted by described algorithms (Frishman and Argos, 1997).

b) Epitope-tagged SUV39H1 proteins in HeLa cells

5 The HeLa cell lines overexpressing full-length (myc)₃-SUV39H1 (aa 3-412) or (myc)₃-Nchromo (aa 3-118) have been described (Aagaard *et al.*, 1999; Melcher *et al.*, 2000). Nuclear extracts were immunoprecipitated with anti-myc antibody beads (Aagaard *et al.*, 1999), and approximately 1-3 µg of matrix-bound (myc)₃-tagged SUV39H1 proteins were used for *in vitro*
10 HMTase assays.

c) Generation and purification of GST-fusion proteins

The GST-Suv1(82-412) product expressed from the pGEX-2T vector (Pharmacia) as a glutathione-S-transferase (GST) fusion protein has been described (Aagaard *et al.*, 1999). Additional GST constructs were
15 generated by transferring BamHI-EcoRI PCR amplicons into pGEX-2T, encoding in-frame fusions for Suv39h1(7-221), SUV39H1(82-412), SUV39H1(82-378) ΔC-tail, SUV39H1(255-412) Δcys, Suv39h2(157-477) (O'Carroll *et al.*, 2000), CLR4(127-490) (Ivanova *et al.*, 1998), EZH2(382-747) (Laible *et al.*, 1997) and HRX(3643-3969) (Tkachuk *et al.*, 1992).
20 Short internal deletions (ΔNHSCDPN₃₂₃₋₃₂₉ and ΔGEELTFDY₃₅₈₋₃₆₅) or point mutations within the -320HφφNHSC₃₂₆- motif were directly engineered in the GST-SUV39H1(82-412) plasmid by double PCR mutagenesis. All constructs were confirmed by sequencing.

Recombinant proteins were expressed in 1l cultures of *E.coli* strain BL21
25 and solubilized in 10 ml RIPA buffer (20 mM Tris pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate) containing a full set of protease inhibitors (Boehringer Mannheim) and lysozyme (5 mg/ml; Sigma)

by freeze-thawing in liquid N₂, followed by sonication. Soluble proteins were cleared by centrifugation, purified with 800 µl glutathione Sepharose beads (Pharmacia) and washed twice in RIPA buffer. Protein concentration was determined by Coomassie staining of SDS-PAGE gels. Matrix-bound
5 fusion proteins were used immediately for *in vitro* HMTase assays or stored at 4°C.

d) *In vitro* histone methyltransferase (HMTase) assay

In vitro HMTase reactions were modified based on described protocols (Strahl *et al.*, 1999) and carried out in a volume of 50 µl of methylase
10 activity buffer (MAB: 50 mM Tris pH 8.5, 20 mM KCl, 10 mM MgCl₂, 10 mM β-ME, 250 mM sucrose), containing 10 µg of free histones (mixture of H1, H3, H2B, H2A and H4; Boehringer Mannheim) as substrates and 300 nCi S-adenosyl-[methyl-¹⁴C]-L-methionine (25 µCi/ml) (Amersham) as methyl donor. 10 µg of matrix-bound GST-fusion proteins were routinely used to
15 assay for HMTase activity. After incubation for 60 min. at 37°C, reactions were stopped by boiling in SDS loading buffer, and proteins were separated by 15% or 18% SDS-PAGE and visualised by Coomassie staining and fluorography.

HMTase assays with individual histones (Boehringer Mannheim), insulin
20 (Sigma) or N-terminal peptides were performed with 5 µg of substrate. The following peptides were used: wild-type N-terminus of human histone H3 (ARTKQTARKSTGGKAPRKQL) (SEQ ID NO:7) and mutant peptide which changes lysine 9 (bold) to leucine; N-terminus of human CENP-A (MGPRRRSRKPEAPRRRSPSP) (SEQ ID NO:8) (Sullivan *et al.*, 1994);
25 N-terminus of rat macro-H2A (MSSRGGKKKSTKTSRSAKAG) (SEQ ID NO:9) (Pehrson and Fried, 1992).

Peptide microsequencing of the *in vitro* methylated wild-type H3 N-terminal peptide and determination of ^3H -incorporation of individual amino acids by scintillation counting was done as described (Strahl *et al.*, 1999).

e) Targeting of the *Suv39h1* and *Suv39h2* gene loci in embryonic stem
5 cells

Partial genomic clones of the *Suv39h1* locus (X chromosome) and of the
Suv39h2 locus (chromosome 2) (O'Carroll *et al.*, 2000) were used to
generate short and long arms of homology, in a strategy to produce in-
frame fusion proteins of the first 40 amino acids of *Suv39h1* or of the first
10 113 amino acids of *Suv39h2* with β -galactosidase (LacZ) modified with a
nuclear localization signal (nls). For targeting, a 1.2 kb Pfu PCR amplicon
and a 5.4 kb *SacI* DNA fragment were derived from the genomic subclone
gSuv39h1 #18, and a 1.3 kb Pfu PCR amplicon and a 5.0 kb *MluI*/*Apal*
DNA fragment were prepared from the genomic subclone *gSuv39h2* #28
15 (see Figure 9A). The pGNA-derived targeting cassettes contained an RSV-
neomycin (neo) gene for positive selection and two polyadenylation sites.
The *diphtheria toxin A* (DTA) gene under the control of the MCI promoter
was used to select against random integration and was inserted 3' of the
long arms of homology. After linearisation with *NotI*, *Suv39h1* and *Suv39h2*
20 targeting constructs were electroporated into feeder-dependent R1 and
E14.1 (129/Sv) embryonic stem (ES) cells.

After selection, G418-resistant ES cell colonies were screened for
homologous recombination by nested PCR using primers external to the
short arms of *Suv39h1* (PCR1: 5'-ATGGGGGCAGGGTTTTCGGGTAGAC,
25 SEQ ID NO:10; PCR2: 5'-AAATGGTATTTGCAGGCCAC-TTCTTG,
SEQ ID NO:11) or of *Suv39h2* (PCR1: 5'-GAAAAGGTTGTTCTCCAGCTC,
SEQ ID NO:12; PCR2: 5'-GGATGGGATGGTGG-AATGGTTTTTAT,
SEQ ID NO:13) and primers within the *lacZ* gene (*lacZ*-PCR1: 5'-
AACCCGTCGGATTCTCCGTGGGAAC, SEQ ID NO:14; *lacZ*-PCR2: 5'-

CTCAGGAA-GATCGCACTCCAGCC, SEQ ID NO:15).

Successful targeting was confirmed by Southern blot analysis of *PvuII*-digested ES cell DNA with a \approx 500 bp external *Suv39h1* intron probe, generated with the primers g24r (5'-GACTGC-CTAGTCTGGCACTGAACT, SEQ ID NO:16) and g13 (5'-GATCACTGCGTACATATAC-ACTGAT, SEQ ID NO:17), or of *HindIII*-digested ES cell DNA with a \approx 500 bp external *Suv39h2* exon/intron probe, generated with the primers P1f (5'-TAGACTT-CTACTACATTAACG, SEQ ID NO:18) and P1r (5'-GATGTCAGTGGCTATGAATG, SEQ ID NO:19). These DNA probes detect a 4.5 kb fragment from the wildtype *Suv39h1* allele and a 4.0 kb fragment from the targeted allele, or 11 kb and 6.1 kb fragments from the *Suv39h2* wildtype and targeted alleles (see Figure 9B).

f) Generation and genotyping of *Suv39h1*- and *Suv39h2*-deficient mice

Several independently targeted ES cell clones gave rise to chimaeric mice which passed the mutations through the germline. *Suv39h1*^{-/-} and *Suv39h2*^{-/-} mice were intercrossed to produce compound *Suv39h* mutant mice (e.g. *Suv39h1*^{-/-}, *Suv39h2*^{+/-}; null1/het2), which were then mated to generate *Suv39h* double null (dn) mice. All mice described in this study were maintained on a mixed genetic background of 129/Sv and C57Bl/6J origin.

Genotyping of mutant mice was done by Southern blot analysis as described above. Protein blot analysis of nuclear extracts from mouse testes with α -*Suv39h1* and α -*Suv39h2* antibodies was performed as described previously (O'Carroll *et al.*, 2000).

g) Generation and analysis of *Suv39h* double null primary mouse embryonic fibroblasts (PMEFs)

PMEFs were derived from day E12.5 *Suv39h* double null embryos obtained after intercrossing *Suv39h1*^{-/-}/*Suv39h2*^{+/-} compound mutant mice. As controls, PMEFs were prepared from wild-type embryos of the same genetic background. For cell cycle profiles and growth curve
5 analysis, passage 2 PMEFs were analyzed as described (Xu *et al.*, 1999). Staining of PMEF interphase chromatin with α -phosphH3 (Hendzel *et al.*, 1997) antibodies was done in unpermeabilized cells as described (Melcher *et al.*, 2000). For the biochemical analysis, total nuclear extracts were precalibrated by Ponceau staining, immuno-blotted with α -H3 (Upstate
10 Biotechnology) and α -phosphH3 (Hendzel *et al.*, 1997) antibodies and visualised by peroxidase staining using Enhanced ChemiLuminescence (ECL) (Amersham).

h) Growth curves and FACS analyses of PMEFs

To analyze the proliferative potential of wild-type and mutant cells, PMEFs
15 were seeded onto 10 cm² dishes. Over the next 30 passages, 3x10⁵ cells were continually reseeded every third day onto a new 10 cm² dish (3T3 protocol), and their doubling rates determined. The DNA profiles of passage 3 and passage 8 PMEF cultures were obtained by FACS of ethanol-fixed and propidium-iodide stained cells, using chicken erythrocyte
20 nuclei (Becton Dickinson) as an internal standard.

i) Bone marrow culture and FACS analysis of B-cell lymphoma cells

Bone marrow cells from wt and *Suv39h* dn mice were cultivated for two weeks in StemPro-34 SFM medium (Life Technologies) supplemented with IL-3 (10 ng/ml), IL-6 (5 ng/ml), SCF (100 ng/ml), FLT 3 ligand (20 ng/ml),
25 GM-CSF (1 ng/ml) (all from R&D Systems), 10 μ M dexamethasone (Sigma) and IGF-1 (40 ng/ml) (Sigma). Cultures were grown at densities of \approx 3x10⁶ cells per ml, and purified from differentiated and dead cells by Ficoll-Paque gradient centrifugation (Pharmacia).

Primary lymphoma cells were obtained from spleen and lymph nodes using a 70 μ m Nylon Cell Strainer (Becton Dickinson), and cultivated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine and 1% penicillin-streptomycin
5 (all Gibco-BRL). Single cells suspensions were grown O/N in medium additionally containing 50 μ M β -mercaptoethanol and 5% conditioned supernatant from rIL-7 producing J558L cells.

The identity of the tumor cells was determined by FACS analyses using antibodies (all from Pharmingen) that detect specific cell surface markers.
10 All tumor cells were double positive for the B-cell markers B220-low (RA3-6B2) and CD19 (1D3), but negative for the T-cell markers CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), or for the granulocyte/macrophage markers Gr-1 (RB6-8C5), Mac-1 (M1/70) and for a marker of the erythroid lineage, Ter-119. The majority of the B-cell lymphoma cells
15 were also double positive for CD43 (S7) and IgM (R6-60.2), while some clonal cultures displayed reactivity towards CD5 (53-7.3). These FACS profiles characterize the Suv39h-mediated tumors as being similar to chronic lymphoid leukemia in humans (Foon and Gale, 1995).

j) Chromosome spreads and karyotype analyses

20 PMEF and tumor cell karyotypes were analyzed on colchicine-arrested and Giemsa-stained metaphase chromosome spreads.

Metaphase spreads of spermatogonia and spermatocytes were prepared from isolated seminiferous tubule fragments which had been hypotonically swollen with 1% sodium citrate for 10 min. at RT and fixed O/N at 4°C with
25 Carnoy's solution (75% methanol, 25% acetic acid). After incubation of seminiferous fragments in 60% acetic acid for 2 min., a single cell suspension was generated by repeated pipetting, transferred onto a pre-heated (60°C) glass slide, and cells were spread by mechanical shearing

with a glass hockey stick.

k) Generation and purification of α -methH3-K9 antibodies

To generate methyl-specific antibodies against the histone H3 lysine 9 position, a hexameric peptide was generated, -TARK(Me)₂ST-cys, containing a di-methylated lysine (Bachem) and a terminal cysteine. To increase the antigenicity and immunogenicity, a 'branched' peptide that consists of four -TARK(Me)₂ST- 'fingers' which are linked at their C-termini via lysine residues was also synthesized. The sequence of this 'branched' peptide is [TARK(Me)₂ST]₄-K₂-K-cys. Peptides were coupled to KLH and rabbit polyclonal antisera were raised, indicating that the 'branched' peptide was much more immunogenic than the linear peptide.

Crude antisera from two positive rabbits (#2233 and #2236) were batch-absorbed against a 'branched', but unmodified control peptide, followed by affinity purification against the di-methylated 'branched' antigen that had been crosslinked to a Poros™ column (Lachner *et al.*, 2001). Bound antibodies were eluted with 100 mM glycine pH 2.5 and neutralised with 1/10 vol. of 2 M Hepes pH 7.9. The methyl-specificity of the antibodies was confirmed on slotblots presenting unmodified or K9-dimethylated histone H3 peptides and on protein blots containing nuclear extracts from wt or *Suv39h* dn PMEFs. The affinity-purified α -methH3-K9 antibodies (concentration \approx 0.6 mg/ml) can be used at a 1:1,000 dilution for protein blot analysis or at 1:1,000 to 1: 5,000 dilutions for indirect immunofluorescence.

l) Immunofluorescence of interphase chromatin and metaphase chromosomes

Passage 6 PMEFs were fixed with 2 % p-FA for 10 min. on ice, washed, incubated with blocking solution (PBS, 2.5 % BSA, 10 % goat serum and

0.1 % Tween20) for 30 min at RT and stained O/N at 4°C with the α -methH3-K9 antibodies. After several washes with PBS containing 0.2 % BSA and 0.1% Tween20, the primary antibodies were detected with Alexa Fluor488-conjugated goat α -rabbit antibodies (Molecular Probes). DNA was
5 counterstained with 4',6'-diamidino-2-phenylindole (DAPI), and samples were embedded in Vectashield (Vector Laboratories).

For preparation of metaphase chromosomes, bone marrow cells or primary tumor cells were arrested by colchicine treatment (0.5 mg/ml) (Sigma) for 2.5 hrs., followed by hypotonic swelling in 0.6 % KCl or RBS buffer (10 mM
10 TrisHCl pH 7.4; 10 mM NaCl; 5 mM MgCl₂) for 15 min. at 37°C and centrifugation for 8 min. at 2000 rpm in a Cytospin (Shandon). Spreaded cells were immediately fixed with icecold 2% p-FA in PBS for 15 min., washed twice and stained with the α -methH3-K9 antibodies as described above.

15 m) Testes histology

Testes were dissected from adult mice, fixed in Bouins fluid (75 % saturated picric acid, 5 % glacial acetic acid, 9.3 % formaldehyde) and stained with haematoxylin/eosine. Staging of the seminiferous tubules was performed according to Oakberg (1956) and Russell *et al.* (1990). FISH
20 analyses with mouse major satellite DNA probes were done as recently described (Scherthan *et al.*, 1996), and Tunel assays were performed using the DeadEnd apoptosis detection system (Promega). In addition, testis cryosections (O'Carroll *et al.*, 2000) were also analyzed by immuno-
25 histochemistry with α -Scp, α -Hp1 β , α -phosH3 and α -meth H3-K9 antibodies.

n) Immunofluorescence of germ cells and meiotic chromosome spreads

Chromosome spreads of spermatogenic cells were prepared according to Peters et al. (1997a) with some minor modifications. A single germ cell suspension was obtained in DMEM medium by mechanical disruption of isolated seminiferous tubules. After several washes and hypotonic swelling in hypobuffer (30mM TrisHCl pH 8.2, 50 mM sucrose, 17 mM sodium citrate) for 10 min. at RT, cells were resuspended in 100 mM sucrose, 15 mM TrisHCl pH 8.2 and spreaded on precleaned slides covered by a thin film of 1% p-FA containing 5 mM borate pH 9.2 and 0.15% TritonX-100. Slides were dried slowly in a humid chamber for \approx 2 hrs and stored at -80°C . Classification of meiotic sub-stages was performed according to the changing morphology of autosomes and sex chromosomes as described (Peters et al., 1997b).

Double-labelling immunofluorescence of these germ cell preparations was performed by sequential incubation with rabbit polyclonal α -methH3-K9 antibodies and with goat α -rabbit Alexa568-conjugated secondary antibodies. After a brief fixation in 1% p-FA, samples were incubated with rabbit polyclonal α -Scp3 antibodies (Lammers et al., 1995) that were visualized with goat α -rabbit Alexa488-conjugated secondary antibodies. In addition, co-stainings were also done with α -Scp3 and α -Scp1 (Offenberg et al., 1991) (see Figure 14A-C), and α -Scp3 and α -HP1 β (Wreggett et al., 1994), and α -Scp3 and α -phosH3 (Hendzel et al., 1997) antibodies.

o) EM analysis

Preparation and silver staining of SC complexes from spreaded germ cells (see above) was performed according to Peters et al. (1997a), and samples were analyzed on a Jeol 1200 EKII transmission electron microscope.

Example 1

Sequence similarity of SET domains with plant methyltransferases

5 Using the SET domains of the SU(VAR)3-9 protein family as a starting alignment, significant sequence and secondary structure similarities (see Methods) to six plant protein methyltransferases were detected. Although some of these plant sequences have been classified as potential histone lysine N-methyltransferases, only one had been functionally characterised
10 but was found to lack HMTase activity (Klein and Houtz, 1995; Zheng *et al.*, 1998).

Detected were amino acid and secondary structure [β -sheet (b) or α -helix (h)] similarities of the C-terminal halves of SET domain sequences from human SUV39H1 (Aagaard *et al.*, 1999) (AF019968), murine Suv39h1
15 (Aagaard *et al.*, 1999) (AF019969), murine Suv39h2 (O'Carroll *et al.*, 2000), (AF149205), *Drosophila* SU(VAR)3-9 (Tschiersch *et al.*, 1994) (P45975), a *C.elegans* SU(VAR)3-9-like ORF C15H11.5 (CAB02737), *S.pombe* CLR4 (Ivanova *et al.*, 1998) (O74565), human EZH2 (Laible *et al.*, 1997) (Q15910), the human trithorax homologue HRX (Tkachuk *et al.*,
20 1992) (Q03164), and MTases from *P.sativum* (rubisco ls-MT; Q43088) (Klein and Houtz, 1995; Zheng *et al.*, 1998) and *A.thaliana* (O65218). The plant MTase sequences contain an insertion of approximately 100 amino acids in the middle of the SET domain.

Example 2

HMTase activity of transfected and recombinant SUV39H1 and Suv39h1 proteins

To investigate whether the SET domain of human SUV39H1 has enzymatic activity, histones were tested as possible substrates for *in vitro* methylation. Using HeLa cell lines 'stably' expressing triple myc-tagged full-length SUV39H1 (aa 3-412), the ectopic protein was enriched from nuclear extracts by immunoprecipitation with anti-myc beads (see Figure 1/1A, arrowhead top panel) and probed for activity to transfer a labelled methyl group from S-adenosyl-[methyl-¹⁴C]-L-methionine to free histones according to described conditions (Strahl *et al.*, 1999). Reaction products were separated by SDS-PAGE and visualised by fluorography, indicating selective transfer of the methyl-label to H3 (Figure 1/1A, lower panel). By contrast, no signals were detected with extracts from a HeLa cell line that expresses only the N-terminal third of SUV39H1 (aa 3-118) or with extracts from HeLa control cells. To confirm that the HMTase activity is an intrinsic property of SUV39H1 and not mediated by a SUV39H1-associated factor, the *in vitro* HMTase reactions was repeated with recombinant products that were purified as GST-fusion proteins from *E.coli* (see Figure 1/2B, arrowheads top panel). For this analysis, murine Suv39h1, which is 95% identical to human SUV39H1 (Aagaard *et al.*, 1999) was used. A purified GST-product comprising aa 82-412 maintained HMTase activity (although at a reduced level as compared to transfected SUV39H1), whereas a purified GST-product comprising aa 7-221 proved negative, even at higher protein concentrations (Figure 1/2B, lower panel). These results suggest that the HMTase activity resides in the C-terminal SET domain.

In Fig. 1A, triple myc-tagged full-length human SUV39H1 (aa 3-412) or a C-terminally truncated SUV39H1 protein (aa 3-118) were

immunoprecipitated from 'stably' transfected HeLa cell lines with anti-myc antibody beads and used in *in vitro* HMTase reactions with free histones as substrates and S-adenosyl-[methyl-¹⁴C]-L-methionine as methyl donor. The Coomassie stain (top panel) shows purified proteins by arrowheads and free histones by dots. Fluorography (bottom panel, Figure 1A) indicates HMTase activity of (myc)₃-SUV1(3-412).

In the experiments shown in Fig. 1B, recombinant GST-fusion proteins encoding different domains of murine Suv39h1 were used in increasing protein concentrations for *in vitro* HMTase reactions as described above.

10

Example 3

a) Definition of a catalytic motif in the SET domain of human SUV39H1

Similar to the recombinant murine GST-Suv39h1(82-412) product, the corresponding human SUV39H1 fusion protein [GST-SUV39H1(82-412)] is catalytically active (see Figure 2). Short internal deletions (Δ NHSCDPN₃₂₃₋₃₂₉; Δ NHSC and Δ GEELTFDY₃₅₈₋₃₆₅; Δ GEEL) were introduced into the two conserved regions of the SET domain core in GST-SUV39H1(82-412) and, in addition, mutants that lack the C-terminal tail (Δ C-tail) or the SET-associated cysteine-rich region (Δ cys) were also generated. All mutant proteins failed to demonstrate HMTase activity (see Figure 2/1A). To investigate enzyme function of the SET domain in more detail, point mutations were introduced into the most highly conserved motif. *In vitro* HMTase assays indicated that all point mutations, with the exception of one, abolished enzymatic activity. Surprisingly, the latter mutation (H320R) resulted in an hyperactive enzyme with approximately 20-fold increased activity. The data obtained define the 320H ϕ ϕ NHSC₃₂₆ motif in the SET domain as an important catalytic site.

b) Specific HMTase activity of the SET domain of mammalian
SU(VAR)3-9 related proteins

Because the SET domain is one of the most conserved protein motifs in chromatin regulators (Stassen *et al.*, 1995; Jenuwein *et al.*, 1998), it was
5 next analyzed whether SU(VAR)3-9 family members or other SET domain proteins contain HMTase activity. GST-fusion products of the extended SET domains of *S.pombe* CLR4 (Ivanova *et al.*, 1998), human EZH2 (Laible *et al.*, 1997) and human HRX (Tkachuk *et al.*, 1992) were generated that would correspond to GST-SUV39H1(82-412) (see Figure 2/2B).
10 Interestingly, GST-CLR4(127-490) displayed pronounced HMTase activity at three- to five-fold increased levels (see Figure 2/2C) as compared to the recombinant SUV39H1 product, consistent with CLR4 carrying an arginine at the hyperactive position. By contrast, both GST-EZH2(382-747) and GST-HRX(3643-3966) had undetectable HMTase activity towards free
15 histones (Figure 2/2C), whereas a comparable GST product generated from the recently isolated murine *Suv39h2* gene (O'Carroll *et al.*, 2000), GST-Suv39h2(157-477), was as active as GST-SUV39H1(82-412). EZH2 lacks the C-terminal cysteines, and HRX does not contain the SET associated cysteine-rich region (Figure 2B). Both of these cysteine
20 domains are present in CLR4, Suv39h2 and SUV39H1. In agreement with the mutational analysis of SUV39H1, it thus appears that HMTase activity towards free histones requires the combination of the SET domain with adjacent cysteine-rich regions, and is a quality found in only a restricted number of SET domain containing proteins.

25 In Fig. 2A, approximately 10 µg of the indicated fusion proteins encoding GST-SUV1(82-412) (= human SUV39H1) and seven SET domain mutants were used in *in vitro* HMTase reactions with free histones as outlined in Figure 1. For the hyperactive H320R mutant, only 1 µg (10%) of the corresponding fusion product was used. Fig. 2 shows a diagram

representing the domain structures of CLR4, Suv39h2, SUV39H1, EZH2 and HRX proteins, with the arrowheads demarcating the N-terminal fusion to GST. Cysteine-rich regions are indicated by grey stippling.

In Fig. 2C, approximately 10 µg of the indicated fusion proteins encoding
5 *S.pombe* CLR4 [GST-CLR4(127-490)], murine Suv39h2 [GST-Suv2(157-477)], human EZH2 [GST-EZH2(382-747)], human HRX [GST-HRX(3643-3969)] and human SUV39H1 [GST-SUV1(82-402)] were used in *in vitro* HMTase reactions with free histones as outlined in Figure 1.

10 Example 4

Lysine 9 of the H3 N-terminus is the major site for *in vitro* methylation by recombinant Suv39h1

The above Examples indicated that the HMTase activity of mammalian SU(VAR)3-9 related proteins is selective for H3 under the chosen assay
15 conditions. To examine this finding in more detail, *in vitro* methylation reactions were performed with individual histones, using GST-Suv39h1(82-412) as an enzyme. As shown in Figure 3/1A, H3 is specifically methylated by GST-Suv39h1(82-412), whereas no signals are detected with H2A, H2B or H4. A weak signal is present if H1 was used as
20 the sole substrate; the significance of H1 methylation remains to be determined. Methylation of H3 has been shown to occur predominantly at lysine 4 in a wide range of organisms, as well as at lysine 9 in HeLa cells, although the responsible HMTase(s) have yet to be defined (Strahl *et al.*, 1999). To investigate the site utilisation profile of Suv39h1, unmodified
25 peptides comprising the wild-type H3 N-terminus (aa 1-20) and a mutant K9L peptide, changing lysine 9 to leucine were tested as substrates. Additionally, insulin and peptides comprising the N-termini of CENP-A (Sullivan *et al.*, 1994) and macroH2A (Pehrson and Fried, 1992) were

included. Peptides were *in vitro* methylated by GST-Suv39h1(82-412), and reaction products were separated by high percentage SDS-PAGE and visualised by fluorography. These *in vitro* assays revealed selective methylation of the wild-type H3 peptide, whereas no signals were detected
5 with the CENP-A or macroH2A peptides, or with insulin (see Figure 3/2B). Importantly, the mutated H3 (K9L) peptide was not a substrate, suggesting that lysine 9 of the H3 N-terminus is a preferred residue for Suv39h1-dependent HMTase activity.

To more definitively determine this site preference, the wild-type H3
10 N-terminal peptide was *in vitro* methylated by GST-Suv39h1(82-412), using S-adenosyl-[methyl-³H]-L-methionine. The labelled peptide, purified by reverse-phase HPLC, was then directly microsequenced, and ³H-incorporation associated with each individual amino acid was analysed by scintillation counting. The results confirmed selective transfer of methyl-
15 label to lysine 9 (see Figure 3/2C), demonstrating that Suv39h1 is a highly site-specific HMTase for the H3 N-terminus *in vitro*.

In Fig. 3A, approximately 10 µg of murine GST-Suv39h1(82-412) were used in *in vitro* HMTase reactions with individual histones as outlined in Figure 1.

20 Fig 3B shows the results of *in vitro* methylation assays using GST-Suv39h1(82-412) as enzyme and the indicated N-terminal peptides of wild-type H3, mutated H3 (K9L), CENP-A, macroH2A or insulin as substrates.

Fig 3C shows the result of automated sequencing of the wild-type H3 N-terminal peptide (aa 1-20) that had been methylated *in vitro* by
25 recombinant GST-Suv39h1(82-412). Displayed is the ³H-incorporation of individual amino acids identified at each successive round of microsequencing.

Example 5

Targeting the *Suv39h1* and *Suv39h2* loci in the mouse germline

Murine *Suv39h* genes are encoded by 2 loci, *Suv39h1* and *Suv39h2* (O'Carroll *et al.*, 2000). To investigate the *in vivo* significance of *Suv39h* function and *Suv39h*-dependent H3-K9 methylation, mouse strains deficient for both *Suv39h1* and *Suv39h2* were generated according to standard techniques. The targeting strategies are shown in Figure 4, as well as demonstrating the production of null alleles for both *Suv39h1* and *Suv39h2*. Mutation of either gene results in viable and fertile mice as a consequence of functional redundancy between both loci. Therefore, *Suv39h1* and *Suv39h2* deficient strains were intercrossed to produce *Suv39h* double null mice. *Suv39h* double null mice are born in sub-Mendelian ratios (see Example 8B, below), where only approximately 30% of the expected *Suv39h* double mutants are observed. Fig. 4 shows a conventional targeting strategy used to inactivate the X-linked *Suv39h1* locus. Fig. 4B shows the Northern blot analysis of *Suv39h1* from spleen (Sp), liver (Li), kidney (Kidney), and brain (Br) from wild-type and *Suv39h1* null mice. Fig. 4C shows the conventional targeting strategy used to inactivate the autosomal *Suv39h2* locus. (Bottom panel) Western blot analysis with α -*Suv39h2* antibodies on protein extracts derived from wild-type and *Suv39h2* null testis.

Example 6

Aberrant mitoses in *Suv39h* double null primary mouse fibroblasts (PMEFs)

In order to determine whether the embryonic phenotypes in *Suv39h* double null mice can be attributed to mitotic defects, PMEFs derived from *Suv39h* double mice were analysed. Cell cycle profiles of wild-type and *Suv39h*

double null PMEFs indicated rather similar percentages of cells to be in S- and G2/M-phases (see Figure 5A), whereas *Suv39h* double null PMEFs display a reduced G1-index and an increased proportion of cells with aberrant nuclear morphologies, reminiscent of division defects during mitosis. For example, *Suv39h* double null PMEFs contain approximately two-fold elevated numbers of cells with micro- and polynuclei, and are further characterised by cell subpopulations with oversized nuclei or a weak definition of heterochromatin that appears in only a few unusually condensed foci (see Figure 5B). Furthermore, *Suv39h* double null cells also show genomic instabilities and readily become aneuploid (see also Example 9, below). The severity of these aneuploidies increases with higher passage numbers (see Figure 6). The inability of *Suv39h* double null cells to maintain a stable karyotype may underlie the *Suv39h* embryonic phenotype.

Fig. 5A shows the percentages of cells in various cell cycle stages of wild-type and *Suv39h* double null PMEFs.

Fig. 5B shows representative images (left and middle) of aberrant mitoses in *Suv39h* double null PMEFs detected by α -tubulin (not shown) and DAPI staining. Also shown (right image) is a nucleus exemplifying the unusual definition of heterochromatin in a subpopulation of *Suv39h* double null PMEFs. All images were taken at a magnification of 630x.

Fig. 6A shows the DNA content profile of wild-type and *Suv39h* double null PMEFs at passage 3. Fig. 6B shows the DNA content profile of wild-type and *Suv39h* double null PMEFs at passage 8.

Example 7

Increased phosH3 phosphorylation in *Suv39h* double null PMEFs

Phosphorylation at serine 10 (phosH3) in the N-terminal tail of H3 has been shown to be required for condensation and subsequent segregation of chromosomes (Wei *et al.*, 1999). During the cell cycle, phosH3 initiates within pericentric heterochromatin in late G2 and then progresses along the entire chromosomes during mitosis (Hendzel *et al.*, 1997). In wild-type PMEFs, approximately 7% of the cells stain positive for the characteristic, heterochromatin-associated phosH3 foci, as detected by indirect immunofluorescence with α -phosH3 antibodies (see Figure 7A, right panel). In contrast, this number is increased by a factor of about 3-fold in *Suv39h* double null PMEFs, with approximately 22% of the cells containing phosH3-positive foci (Figure 7A, left panel), although their definition appears in many small speckles which do not always overlap with DAPI-dense material. This result suggested that the overall levels of phosH3 may be enhanced in *Suv39h* double null PMEFs. Therefore, the relative abundance of phosH3 in precalibrated nuclear extracts was determined with α -phosH3-specific antibodies. This quantitation indicated a significantly higher level of phosH3 in *Suv39h* double null cells as compared to wild-type controls (see Figure 7B). Together, the obtained data are most consistent with a model in which Suv39h-mediated methylation of lysine 9 in H3 negatively regulates phosphorylation of serine 10.

Fig. 7A shows an interphase chromatin staining with α -phosH3 antibodies and CY3-conjugated secondary antibodies. DNA was counterstained with DAPI. At least 1,000 cells were counted to evaluate the percentage (indicated in the Figure) of α -phosH3 positive cells. Fig 7B shows a quantitative Western analysis with 15 μ g and 30 μ g of total nuclear proteins immuno-blotted with α -H3 and α -phosH3 antibodies.

Example 8

a) Generation of *Suv39h* double deficient mice

Murine *Suv39h* HMTases are encoded by two loci which have been
5 mapped to centromere-proximal positions in the X chromosome (*Suv39h1*)
or in chromosome 2 (*Suv39h2*) (O'Carroll *et al.*, 2000). Both gene loci were
independently disrupted by homologous recombination in embryonic stem
(ES) cells using a conventional targeting approach that replaces parts of
the evolutionarily conserved chromo domain with the bacterial *LacZ* gene
10 and an *RSV-neomycin* selection cassette (Figure 9a). These targeting
strategies produce in-frame fusion proteins of the first 40 amino acids of
Suv39h1 or of the first 113 amino acids of *Suv39h2* with *lacZ*, which
maintain β -galactosidase activities. Successfully targeted ES cell clones
were used to generate chimaeric mice that transmitted the mutated
15 *Suv39h1* or *Suv39h2* alleles through the germ line (Figure 9b). Protein blot
analyses of testis nuclear extracts from wild-type, *Suv39h1*- and *Suv39h2*-
deficient mice with α -*Suv39h1* and α -*Suv39h2* specific antibodies (Aagaard
et al., 1999; O'Carroll *et al.*, 2000) indicated the absence of the respective
proteins, demonstrating that had been generated loss-of-function alleles for
20 both genes (Figure 9c).

b) Impaired viability of *Suv39h* double null mice

Mice deficient for either *Suv39h1* or *Suv39h2* display normal viability and
fertility, and do not exhibit apparent phenotypes, suggesting that both
genes may be functionally redundant during mouse development (O'Carroll
25 *et al.*, 2000). Therefore, *Suv39h1*^{-/-} and *Suv39h2*^{-/-} mice were intercrossed
to generate compound *Suv39h* mutants that were then used to derive
Suv39h double null (dn) mice. *Suv39h* dn mice obtained from several
different intercrosses (Table I) are born at only sub-Mendelian ratios, are

growth retarded (Figure 9d) and are characterized by hypogonadism in males. For example, from a total of 197 mice, 46 mice would have been expected to be double null (Table I), but only 15 *Suv39h* dn mice ($\approx 33\%$) were born. Analysis of mouse embryogenesis indicated normal
5 development of *Suv39h* dn fetuses until day E12.5, whereas at later stages, *Suv39h* dn fetuses are smaller and display an increased rate of resorptions and prenatal lethality. Together, these results demonstrate that the *Suv39h* genes are required for normal viability, and for pre- and postnatal development.

10 Figure 9 shows the targeting and genotyping of *Suv39h1*- and *Suv39h2*-deficient mice as follows: (A) Diagrammatic representation of the *Suv39h1* and *Suv39h2* genomic loci, the replacement vectors and the targeted alleles. Exons are indicated by black boxes with numbers referring to the starting amino acid positions of the respective exons (O'Carroll *et al.*,
15 2000). Also shown are the diagnostic restriction sites and the external probes used for Southern blot analyses. pA indicates polyadenylation signals. (B) Southern blot analyses of *PvuII*- or *HindIII*-digested DNA isolated from offspring of *Suv39h1*+/- or *Suv39h2*+/- heterozygous intercrosses. (C) Protein blot analyses of testis nuclear extracts from wild-
20 type (wt), *Suv39h1*-/- (*Suv1*-/-) and *Suv39h2*-/- (*Suv2*-/-) mice with α -*Suv39h1* and α -*Suv39h2* antibodies. The size of the *Suv39h1* or *Suv39h2* proteins is indicated by arrows. (D) *Suv39h* double null (dn) mice are growth retarded at birth and during adulthood.

25 Example 9

Chromosome mis-segregation in *Suv39h* dn embryonic fibroblasts

To examine the *Suv39h*-dependent defects in more detail, primary mouse embryonic fibroblasts (PMEFs) were derived from day E12.5 fetuses.

Comparative growth curves between wild-type (wt) and *Suv39h* dn PMEFs in a 3T3 protocol over the first 20 passages indicated that *Suv39h* dn PMEFs displayed a higher doubling rate until passage 12 (Figure 10a). At later passages, the *Suv39h* dn PMEFs appear to have a slightly reduced proliferative potential than the immortalised wt PMEFs which survived the characteristic Hayflick crisis. It was shown recently (see Example 6) that *Suv39h* dn PMEFs contain a significant fraction of cells with aberrant nuclear morphologies, such as macro- and polynuclei, which are reminiscent of impaired mitosis and chromosome mis-segregation (Rea *et al.*, 2000). Therefore the DNA content of passage 3 and passage 8 wt and *Suv39h* dn PMEFs was analyzed by FACS. Whereas wt PMEFs appear genomically stable at passage 3, *Suv39h* dn PMEFs already contain cells with a greater than 4N DNA content, as indicated by the aneuploid shoulder in the FACS profile (Figure 10B, top panels). At passage 8, wt PMEFs are largely senesced. By contrast, *Suv39h* dn PMEFs continue to proliferate, although many cells display octaploid DNA contents (Fig. 10B, lower panels).

To further characterize these genomic instabilities, karyotype analyses with passage 8 PMEFs were performed (Figure 10C). In particular, 45 karyotypes each for two independent wt and two *Suv39h* dn PMEF cultures were examined. As shown in Figure 10D, a major fraction of the wt karyotypes are non-diploid, with chromosome numbers ranging from 25 to 82. Aneuploidies were significantly increased in *Suv39h* dn karyotypes and comprised chromosome numbers from 38 to 162. Notably, whereas wt PMEFs contain a random array of aneuploid karyotypes, *Suv39h* dn PMEFs are largely hypo-tetraploid or hypo-octaploid. Chromosomes in *Suv39h* dn PMEFs appear of normal morphology and Robertsonian fusions were not observed. It was concluded that the absence of *Suv39h* function induces genomic instabilities, primarily by impairing segregation of the entire set of chromosomes.

Figure 10 shows the chromosomal instabilities in *Suv39h* dn PMEFs as follows: (A) Relative doubling rates of wt and *Suv39h* dn PMEFs determined in a 3T3 protocol over the first 20 passages. (B) DNA contents of wt and *Suv39h* dn PMEF mass cultures at passage 3 and passage 8. (C) 5 Metaphase spreads showing a diploid number ($n = 40$) of chromosomes for wt and a hyper-tetraploid number ($n = 82$) of chromosomes for *Suv39h* dn PMEFs. (D) Statistical karyotype analysis with two wt and two *Suv39h* dn PMEF cultures at passage 8. For each culture, 45 metaphases were evaluated.

10

Example 10

Development of B-cell lymphomas in *Suv39h* mutant mice

Next, *Suv39h* mutant mice were analyzed for the incidence of tumorigenesis. Because the majority of *Suv39h* dn mice are non-viable, 15 distinct *Suv39h* genotypes that differ in their gene dosage for either *Suv39h1* or *Suv39h2* were examined. For example, it was expected that random X-inactivation of the X-linked *Suv39h1* gene could increase the tumor risk in *Suv39h1*+/- mice, even in the presence of a functional copy of *Suv39h2* which is significantly down-regulated in most adult tissues 20 (O'Carroll *et al.*, 2000). Indeed, examination of 98 mice which are either heterozygous (het) or null for the *Suv39h1* locus indicated an $\approx 28\%$ penetrance of tumor formation with an onset between 9 - 15 months of age (Table II). These tumors are predominantly B-cell lymphomas (Figure 11A) that resemble by FACS profiling (see Materials and Methods) slowly 25 progressing non-Hodgkin lymphomas in humans (Foon and Gale, 1995). The tumor incidence for late onset B-cell lymphomas was $\approx 33\%$ in the few viable *Suv39h* dn mice ($n = 6$). By contrast, *Suv39h2*+/- or *Suv39h2*-/- mice developed B-cell lymphomas at only $\leq 5\%$ penetrance ($n = 21$), and tumor

formation in control wild-type mice was not observed.

Primary cultures were derived from the lymph nodes of *Suv39h* dn and of *Suv39h1*^{-/-}, *Suv39h2*^{+/-} (null1/het2) tumor mice, and analyzed the karyotypes of the B-cell lymphoma cells. Consistent with the aneuploides described above for *Suv39h* dn PMEF mass cultures, these tumor cells were largely hyper-diploid but also comprised some hyper-tetraploid karyotypes (Figure 11B). Surprisingly, a fraction of *Suv39h* dn tumor karyotypes, examined in several independent B-cell lymphomas, is characterized by non-segregated chromosomes that remain attached through their acrocentric regions (Figure 11C). These 'butterfly' chromosomes raise the intriguing possibility that the absence of Suv39h HMTase activities could impair the quality and function of pericentric heterochromatin by increasing more persistent interactions between metaphase chromosomes. Indeed, analysis of H3-K9 methylation with a newly developed antibody (see Example 11, below) indicates the absence of methH3-K9 staining at pericentric heterochromatin of tumor chromosomes derived from *Suv39h* null1/het2 B-cell lymphoma cells.

Figure 11 shows the development of B-cell lymphomas in *Suv39h* mutant mice as follows: (A) Spleen and lymph nodes of an 11-month old *Suv39h* dn tumor mouse and of a wild-type control mouse. (B) Karyotype analysis of four independent primary cultures derived from the lymph nodes of tumor-bearing *Suv39h* dn (null1/null2) and *Suv39h1*^{-/-}, *Suv39h*^{+/-} (null1/het2) mice. (C) Metaphase spread from a primary *Suv39h* dn B-cell lymphoma cell showing 'butterfly' chromosomes that remain associated through their acrocentric regions.

Example 11

Absence of H3-K9 methylation at *Suv39h* dn heterochromatin

The above karyotype analyses on PMEF and tumor cells suggested a general mechanism through which segregation of the entire chromosome complement may be impaired by *Suv39h*-dependent defects in pericentric chromatin organization. To assess directly the role of the Suv39h HMTases in histone methylation and heterochromatin formation, a rabbit polyclonal antiserum was raised that specifically recognizes histone H3 when dimethylated at lysine 9 (α -methH3-K9). As shown in Figure 12A, this antiserum detects a focal staining in wt PMEFs that significantly overlaps with DAPI-rich heterochromatin. In PMEFs derived from single *Suv39h1*- or *Suv39h2*-deficient mice, $\approx 75\%$ of cells stain positive for heterochromatic foci with these α -methH3-K9 antibodies. Importantly, heterochromatic staining for methH3-K9 was abolished in *Suv39h* dn PMEFs (Figure 12A, right row).

Mitotic chromosome spreads from bone marrow cells were also analyzed with the α -methH3-K9 antiserum. In wt spreads, pericentric heterochromatin was selectively visualised (see inserts in Figure 12B), whereas only residual staining was detected in *Suv39h* dn spreads. Thus, consistent with the localization of SUV39H1 at active centromeres (Aagaard *et al.*, 2000), these data demonstrate that both Suv39h enzymes are the major HMTases to methylate H3-K9 in pericentric heterochromatin of somatic cells. Moreover, these results also characterize the α -methH3-K9 antibodies as a novel cytological marker for heterochromatin and corroborate recent *S.pombe* studies, in which enrichment of H3-K9 methylation at MAT and CEN regions was shown to be dependent upon a functional Ctr4 enzyme (Nakayama *et al.*, 2001).

Figure 12 shows the *Suv39h*-dependent H3-K9 methylation at pericentric heterochromatin as follows: (A) DAPI and methH3-K9 staining on interphase chromatin of wild-type (wt), *Suv39h1*^{-/-}, *Suv39h2*^{-/-}, and *Suv39h* dn PMEFs. Percentages refer to interphase nuclei displaying H3-K9 methylation at heterochromatic foci. (B) DAPI and methH3-K9 staining on mitotic chromosomes prepared from *in vitro* cultured wt and *Suv39h* dn bone marrow cells.

Example 12

a) Hypogonadism and complete spermatogenic failure in *Suv39h* dn mice

The expression pattern of the *Suv39h* genes suggests an important role during spermatogenesis (O'Carroll *et al.*, 2000). Indeed, *Suv39h* dn males (n = 7) are infertile, do not contain mature sperm and their testis weights are 3 - 10 fold reduced as compared to that of wt males (Figure 13A). To investigate the spermatogenic failure in more detail, histological sections were performed, demonstrating normally developed seminiferous tubules in wt testis which display the characteristic differentiation from the mitotically proliferating spermatogonia (Sg) to meiotic spermatocytes (Sc) and the post-meiotic haploid spermatids (St) (Figure 13A). By contrast, spermatogenesis was severely impaired in *Suv39h* dn mice, with an apparent differentiation arrest at the transition between early to late spermatocytes, resulting in highly vacuolarized seminiferous tubules (Figure 13A).

FISH analyses with mouse major satellite DNA probes and TUNEL assays were used to characterize the *Suv39h*-dependent spermatogenic defects further. Whereas mitotic proliferation of spermatogonia appeared normal, a 3 to 10 fold increase in the percentage of pre-leptotene spermatocytes was observed. These pre-leptotene spermatocytes often were enlarged. These

results suggest that the entry into meiotic prophase is delayed in the absence of *Suv39h* function. Despite this delay, further progression through meiotic prophase until mid-pachytene appeared normal. Between mid- to late pachytene, however, most spermatocytes undergo apoptosis, resulting in stage V - VI tubules (see Figure 13A) that largely lack late pachytene spermatocytes and which do not contain haploid spermatids. It was concluded that the absence of *Suv39h* gene function induces delayed entry into meiotic prophase and triggers pronounced apoptosis of spermatocytes during the mid- to late pachytene stage.

b) H3-K9 methylation at meiotic heterochromatin

To investigate whether the *Suv39h*-dependent spermatogenic failure could be correlated with a distinct impairment of meiotic heterochromatin, testis spread preparations and cryosections were analyzed with the α -methH3-K9 antibodies. In wt preparations, the α -methH3-K9 antibodies decorate heterochromatic foci in spermatogonia (B-Sg) and in pre-leptotene spermatocytes (preL-Sc) (Figure 13B, left images, top panel). In early meiotic prophase (Zyg-Sc) and early pachytene, the α -methH3-K9 staining was not exclusive for heterochromatin but also extended into euchromatin. From mid-pachytene through diplotene and in diakinesis, the α -methH3-K9 staining was restricted to heterochromatic clusters which condense into one block of heterochromatin in elongating spermatids (Figure 13B, top panels). MethH3-K9 signals in elongated spermatids and mature spermatozoa, in which histones are replaced by protamines, were not detect. The authenticity of this staining pattern had been confirmed in co-localisation analyses with antibodies that recognize the synaptonemal complex (Offenberg *et al.*, 1991; Lammers *et al.*, 1995), HP1 β (Motzkus *et al.*, 1999) and phosH3 (Cobb *et al.*, 1999). Thus, in analogy to the somatic stainings shown above for PMEFs, these results indicate that methylation of H3-K9 is also a specific marker for meiotic heterochromatin in

differentiating male germ cells.

c) Impaired H3-K9 methylation and aneuploidies in *Suv39h* dn spermatogonia

In preparations from *Suv39h* dn testis spreads, H3-K9 methylation was
5 absent in spermatogonia and pre-leptotene spermatocytes (Figure 13B, left
images, bottom panel). Further, the pronounced euchromatic staining that
characterizes early spermatocytes (Zyg-Sc) at the onset of meiotic
prophase was not observed. The impairment of H3-K9 methylation was
accompanied by a dispersed distribution of phosH3 in $\approx 60\%$ of *Suv39h* dn
10 spermatogonia. By contrast, HP1 β was largely undetectable in both wt and
Suv39h dn spermatogonia.

Surprisingly, from mid-pachytene onwards, wild-type staining for
methH3-K9 at pericentric heterochromatin was observed (Figure 13B,
bottom panel). HP1 β localisation and phosH3 signals at autosomes occurred
15 normally in *Suv39h* dn late spermatocytes. Thus, these results demonstrate
that the *Suv39h* HMTases selectively regulate H3-K9 methylation in
spermatogonia and at the very early stages of meiotic prophase. Similar to
the analysis with PMEFs (see above), an ≈ 5 -fold increased rate for
complete chromosome mis-segregation in *Suv39h* dn spermatogonia that
20 results in the occurrence of tetraploid spermatocytes was observed (see
Figure 14C, below). In summary, these data define an early and stage-
specific meiotic role for the *Suv39h* HMTases, and further suggest the
existence of a novel H3-K9 HMTase(s) which can methylate
heterochromatin during meiotic prophase, diakinesis and in spermatids.

25 Figure 13 shows the spermatogenic failure and H3-K9 methylation in germ
cells of *Suv39h* dn mice as follows: (A) Overall size and histology of wild-
type and *Suv39h* dn testes at ≈ 5 months of age. The *Suv39h* dn testis
section reveals many seminiferous tubules that lack spermatocytes (Sc)

and spermatids (St). In particular, although a few seminiferous tubules (1) contain zygotene spermatocytes (Zyg-Sc), more advanced differentiation stages (2) display apoptotic spermatocytes (arrows) at pachytene. At even later differentiation stages (3), pachytene spermatocytes are almost
 5 completely absent. Some tubules (4) harbor only Sertoli cells (SeC).
 Abbreviations: Intermediate (In-Sg) and B-type spermatogonia (B-Sg); pre-leptotene (PreL-Sc), zygotene (Zyg-Sc), mid-pachytene (mPach-Sc), late-pachytene (lPach-Sc), diplotene (Diplo-Sc) and diakinesis/M-I (M-I-Sc) spermatocytes; round (rSt), elongating (elSt) and elongated (eSt)
 10 spermatids; Sertoli cells (SeC).

(B) Double-labelling immunofluorescence of wt (top panel) and *Suv39h* dn (bottom panel) germ cells with α -methH3-K9 (pink) and α -Scp3 (green) antibodies. DNA was counterstained with DAPI (blue). In *Suv39h* dn germ cells, H3-K9 methylation is absent in proliferating spermatogonia (B-Sg)
 15 and in pre-leptotene spermatocytes (PreL-Sc), and is highly reduced in zygotene spermatocytes (Zyg-Sc) where only residual signals are detected at pericentric heterochromatin (arrowheads). At later stages, H3-K9 methylation appears in a wild-type staining (compare top and bottom panels), although *Suv39h* dn sex chromosomes (arrows) remain more
 20 intensely labeled at diplotene and diakinesis. The double arrow indicates the pseudo-autosomal region (PAR).

Example 13

a) Non-homologous interactions and delayed synapsis in *Suv39h* dn
 25 spermatocytes

The absence of pericentric H3-K9 methylation in spermatogonia and early spermatocytes is suggestive for a role of the *Suv39h* HMTases in defining a higher-order structure that may be required for the initial alignments and

clustering of meiotic chromosomes. Therefore chromosome synapsis was analyzed by immunofluorescence of pachytene spreads with antibodies that are specific for the axial/lateral and central elements of the synaptonemal complex (SC) (Figure 14A,B). Intriguingly, in $\approx 15\%$ ($n = 90$) of *Suv39h* dn spermatocytes, non-homologous interactions between autosomes were observed (Figure 14J). Non-homologous interactions were even more frequent ($\approx 35\%$) between sex chromosomes and autosomes (X/Y-A). Interestingly, these illegitimate associations occurred predominantly between the acrocentric ends (cen-cen) of non-homologous chromosomes, to a lesser extent between centromeres and telomeres (cen-tel) and only very rarely between telomeres (tel-tel) (Figure 14J). In addition, *Suv39h* dn spermatocytes contained unsynapsed sex chromosomes (see below) and autosomal bivalents that were delayed in synapsis. Delayed synapsis of autosomes (A-del) almost invariably was correlated with engagement in non-homologous associations (Figure 14A), suggesting that both processes may be functionally related.

The illegitimate associations were further confirmed by transmission electron microscopy (Figure 14D-G). These ultrastructural analyses revealed the presence of physical connections and bridge-like structures between the ends of non-homologous chromosomes (double arrow in Figure 14D,C,F). The incidence of partner exchange (Figure 14G) and non-homologous alignments were also observed. None of these aberrant chromosomal interactions were detected in EM preparations from wt spermatocytes.

b) Bivalent mis-segregation at meiosis I in *Suv39h* dn spermatocytes

To determine whether the absence of methH3-K9 in early prophase may affect chromosome dynamics and segregation during the meiotic divisions, testis spread preparations were next analyzed for diakinesis/metaphase I (M-I) and metaphase II (M-II) cells. At diakinesis/M-I, most *Suv39h* dn

spermatocytes revealed bivalents with wt-like morphology, indicating that chromosome condensation and chiasmata formation was unperturbed (but see Figure 15B-D, below). However, at M-II, $\approx 14\%$ of secondary spermatocytes were tetraploid, indicating segregation failure of all bivalents during the first meiotic division (Figure 14I and 14K). Therefore, the *Suv39h*-induced defects at pericentric heterochromatin persist throughout the first meiotic division and do not appear to be 'rescued' by the additional H3-K9 methylation that occurs during mid- to late meiotic prophase (see Figure 13B).

Figure 14 shows the illegitimate associations and delayed synapsis of *Suv39h* dn meiotic chromosomes as follows: (A-C) Double-labelling immunofluorescence of *Suv39h* dn pachytene spermatocytes with antibodies that are specific for the axial/lateral elements α -Scp3 (in green) and central elements α -Scp1 (in red) of the synaptonemal complex (SC). This co-labelling reveals unsynapsed chromosomes in a green-like staining and synapsed chromosomes in an orange-red colour. DNA was counterstained with DAPI (blue) which highlights pericentric heterochromatin in a more intense blue contrast. (A) Two mid-pachytene spermatocytes (mPach-Sc) showing multiple illegitimate associations (arrowheads) between non-homologous autosomes (A) and between autosomes and sex chromosomes (X, Y). Several autosomes are also delayed in synapsis (A_{del}). (B) Late pachytene (lPach-Sc) spermatocyte containing two autosomes which are engaged in non-homologous interaction through their pericentric regions (arrowhead). In addition, the sex chromosomes failed to pair. (C) Tetraploid spermatocyte resulting from complete mis-segregation of all chromosomes in the preceding mitotic division of a *Suv39h* dn spermatogonium.

(D-G) Transmission electron microscopy of *Suv39h* dn pachytene chromosomes, confirming that non-homologous chromosome associations

mainly occur through pericentric heterochromatin which is visualised by the more granular silver staining (arrowhead and double arrows). The chromosomes displayed in panel G show multiple engagements of partner exchange.

- 5 (H, I) Giemsa-stained metaphase II chromosomes of wt and *Suv39h* dn secondary spermatocytes illustrating complete mis-segregation in the preceeding meiosis I division of *Suv39h* dn cells.

- (J) Histogram for the frequency of non-homologous chromosome associations and delayed synapsis in wt (n = 80) and *Suv39h* dn (n = 90) pachytene spermatocytes. (K) Histogram for the frequency of meiosis I mis-segregation of chromosome bivalents in wt (n = 40) and *Suv39h* dn (n = 30) secondary spermatocytes.
- 10

Example 14

- 15 *Suv39h* deficiency interferes with sex chromosome segregation

Spermatogenesis in male mammals is specialised by the presence of the heteromorphic sex chromosomes which form a unique chromatin region known as the sex vesicle or XY body (Solari, 1974). Moreover, the Y chromosome is the most heterochromatic chromosome in the mouse (Pardue and Gall, 1970). Homolog pairing and cross-over between sex chromosomes is dependent upon the presence of a small, pseudo-autosomal region called PAR (Burgoyne, 1982). The absence of *Suv39h* function interferes with the chromatin organization and segregation of the sex chromosomes in several ways.

20

- 25 First, although methH3-K9 signals at the XY body (arrows in Figure 13B) were detected at comparable levels in wt and mutant pachytene spermatocytes, *Suv39h* dn sex chromosomes remain more heavily

5 methylated in diplotene and diakinesis (see Figure 13B, bottom panels). Correspondingly, prolonged HP1 β binding to the XY body during diplotene was observed. Second, at diakinesis/M-I, the proximal region of the long arm of the Y chromosome appears hypo-condensed in 10% of *Suv39h* dn cells (Figure 15B, E). Moreover, the mutant Y chromosomes display premature separation of their arms or even complete separation of the two sister chromatids (Figure 15D, E). Third, H3-K9 methylation is present at the PAR (double arrows in Figure 13B) in both wt and *Suv39h* dn sex chromosomes, and the PAR is also decorated with HP1 β . Despite these similar staining patterns, the sex chromosomes failed to synapse in \approx 15% of *Suv39h* dn pachytene spermatocytes (Figure 14A, B). At diakinesis/M-I (Figure 15B, C), the presence of XY univalents was 4-fold increased as compared to wt cells (Figure 15F). Together, these data indicate a role for the *Suv39h* HMTases in co-regulating the specialised chromatin structure of the sex chromosomes, in particular of the highly heterochromatic Y chromosome.

Figure 15 shows the aberrant function of the Y chromosome during meiosis of *Suv39h* dn spermatocytes as follows: Giemsa-stained diakinesis/metaphase-I chromosomes of wt (A) and *Suv39h* dn (B-D) primary spermatocytes illustrating univalency (B, C), impaired condensation (B, C) and premature sisterchromatid separation of the Y chromosome (C, D). (E) Histogram for the frequency of diakinesis/M-I cells with abnormal condensation or premature sisterchromatid separation of the Y chromosome (wt: n = 190; *Suv39h* dn: n = 170). (F) Histogram for the frequency of XY univalency at pachytene (wt: n = 80; *Suv39h* dn: n = 80) or diakinesis/M-I (wt: n = 190; *Suv39h* dn: n = 170).

Example 15

Screening for modulators of Suv39h1 MTase activity.

All steps are automated and the position of the different test compounds are registered on computer for later reference. Compounds being tested for
5 modulating activity are aliquoted into 384 well plates in duplicate. 20-200 nmol of recombinant GST tagged SUV39H1 in MAB buffer, is then added to the reaction. 20 nmol of branched peptide ([TARKST]4-K2-K-cys) which has been labelled with europium is then added, followed by 100 nmol of S-adenosyl methionine. This reaction is left at room temperature for 40
10 mins, then transferred onto a second plate to which the α -methH3-K9 antibody has been coated. This reaction is then left at room temperature for 40 mins to allow the antibody to bind methylated substrate. Following capture of methylated substrate, unbound non-methylated substrate is washed off in 50 mM tris pH 8.5. The europium label is then cleaved from
15 the peptide in 50 μ l pH 4.5 enhancement solution for 25 mins. The chelated europium molecules are then excited at 360nm and the level of emitted fluorescence at 620nm is then calculated using time-resolved fluorescence in a PolarStar plate reader. The results are then automatically graphed.

The level of fluorescence is directly related to the level of MTase activity.

20 The effect of the different compounds on the MTase activity can be clearly seen on the graph when compared to control reactions with no compounds added or with no enzyme added.

Figure 17 illustrates the principle of the screening method as follows:

- a) A Suv39h1-like MTase is incubated with S-Adenosyl Methionine
25 (SAM) and a chromogenically labelled unmodified peptide substrate (e.g.

branched peptide [TARKST]4-K2-K-cys). Following methylation of this substrate the substrate becomes an epitope for a Lys9-methyl specific antibody which has been immobilised on a microtiter plate. The level of bound peptide can then be quantified by the level of fluorescence of from
5 the chromogenic label.

b) In the presence of a modulator (e.g. an inhibitor, I) the transfer of methyl groups by the MTase will be affected (decreased), this in turn will affect the amount of substrate captured by the immobilised antibody, which is quantified by the level of fluorescence. A compound with inhibitory
10 effects will result in a decrease in fluorescent signal, whereas a compound with enhancing effects will result in an increase in fluorescent signal.

Table I:

Viability of *Suv39h* double null mice.

cross	N1H2 x H1H2 ^a	N1H2 x N1H2	N1H2 x H1N2	total
dn mice expected	1:8	1:4	1:4	
total # mice born	81	89	27	197
# dn mice expected ^b	11	27	8	46
# dn mice observed	4	8	3	15
% dn mice viable	36.4	29.6	37.5	32.6

5 ^a i.e.: N1H2 x H1H2: ♂♂ *Suv39h1*^{-/-}, *Suv39h2*^{+/-} x ♀♀ *Suv39h1*^{+/-}, *Suv39h2*^{+/-}

^b Based on number of mice born with other *Suv39h1* and *Suv39h2* allelic combinations which show no reduced prenatal viability.

Table II:

Incidence of B-cell lymphomas in mice with reduced *Suv39h* gene dosage

Genotype	<i>Suv39h</i> gene dosage	# of mice with tumor	total # of mice	% of mice with tumor
W1W2	3	0	57	0
W1H2, W1N2, H1N2	0 - 2	1	22	4.6
H1W2, N1W2	2 - 3	8	26	30.8
H1H2, N1H2*	1 - 2	20	72	27.8
N1N2	0	2	6	33.3

5 * i.e.: N1H2: *Suv39h1*^{-/-}, *Suv39h2*^{+/-}

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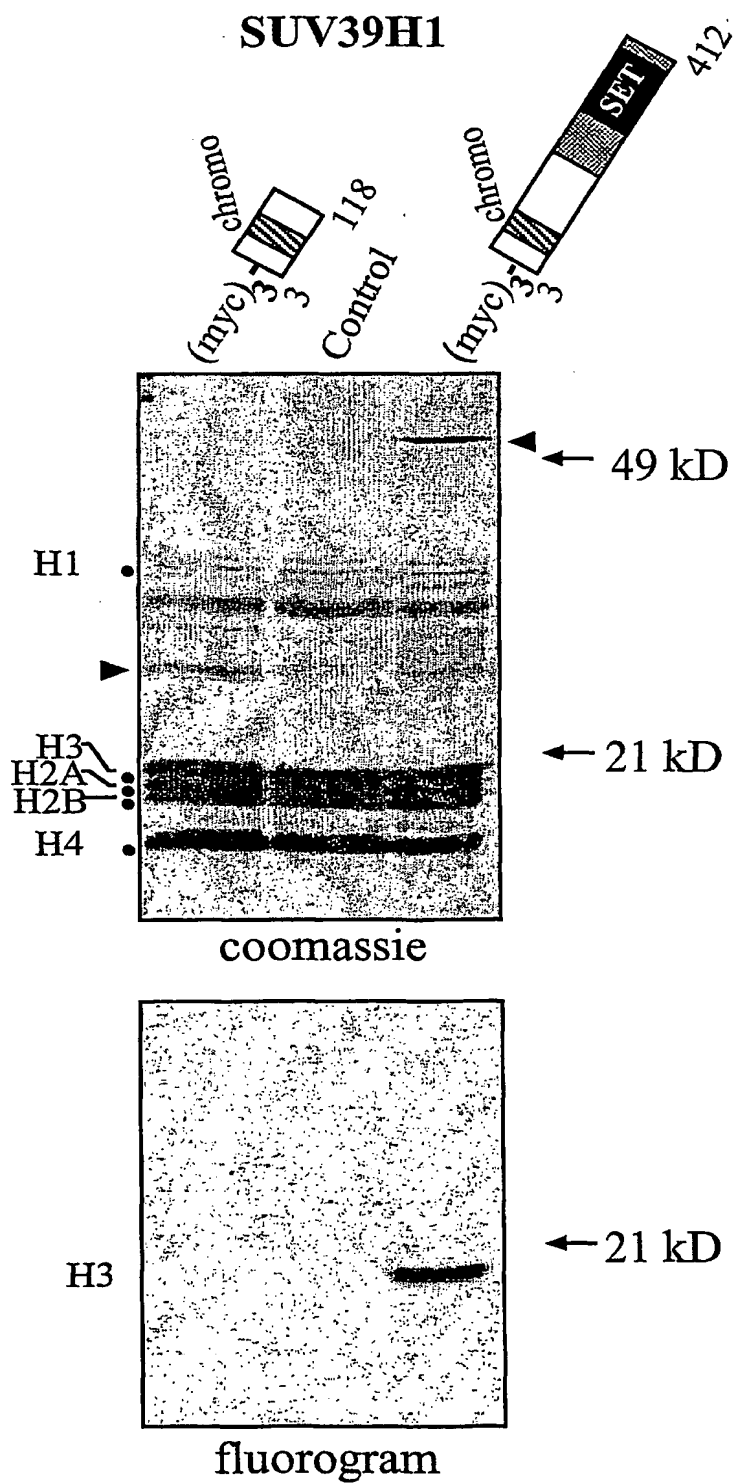
Claims

1. A method for identifying a compound that alters higher order chromatin
5 dependent chromosome stability in mitosis and meiosis, said method
comprising incubating a substrate for a methyltransferase, in the
presence of a methyl donor, with a methyltransferase with Suv39h-like
methyltransferase activity, in the presence or absence of a test
compound and determining whether the compound modulates the
10 methyltransferase activity.
2. The method of claim 1, wherein the methyltransferase with Suv39h-like
activity methylates histone H3 at lysine 9.
3. The method of claim 2, wherein the methyltransferase with Suv39h-like
activity is murine Suv39h1 or human SUV39H1.
- 15 4. The method of claim 2, wherein the methyltransferase with Suv39h-like
activity is murine Suv39h2 or human SUV39H2.
5. The method of any one of claims 1 to 4, wherein the substrate is histone
H3 or an N-terminal fragment thereof that contains the methylation site
at lysine 9.
- 20 6. The method of claim 5, wherein the histone H3 N-terminal fragment has
the amino acid sequence as set forth in SEQ ID NO:7.
7. The method of any one of claims 1 to 6, wherein the methyl donor is
methionine or S-adenosyl-L-methionine.

8. The method of any one of claims 1 to 7, wherein the methyl group of the methyl donor carries a detectable label.
9. The method of claim 8, wherein the methyl donor carries a chromogenic label and the methyltransferase activity is determined by measuring the change in colour upon transfer of the methyl group to the substrate.
10. The method of claim 8, wherein the methyl donor carries a radioactive label and the methyltransferase activity is determined by measuring the radioactivity transferred to the substrate upon transfer of the methyl group.
11. The method of any one of claims 1 to 7, wherein the methyltransferase activity is determined immunologically by quantifying the binding of an antibody specific for the methylation site of the substrate.
12. The method of claim 11 wherein the substrate carries a detectable label.
13. An antibody that specifically recognises methylated lysine 9 in histone H3.
14. The use of the H3-K9 methyl-specific antibody of claim 13 for diagnosis of a human disease associated with aberrant gene expression and genomic instability through chromosome mis-segregation.
15. A compound identified in a method defined in any one of claims 1 to 12 for use in the therapy of cancer.
16. A compound identified in a method defined in any one of claims 1 to 12 for contraception.
17. The compound of claim 15 for use in temporary male conception.

1/33
Fig. 1/1

A

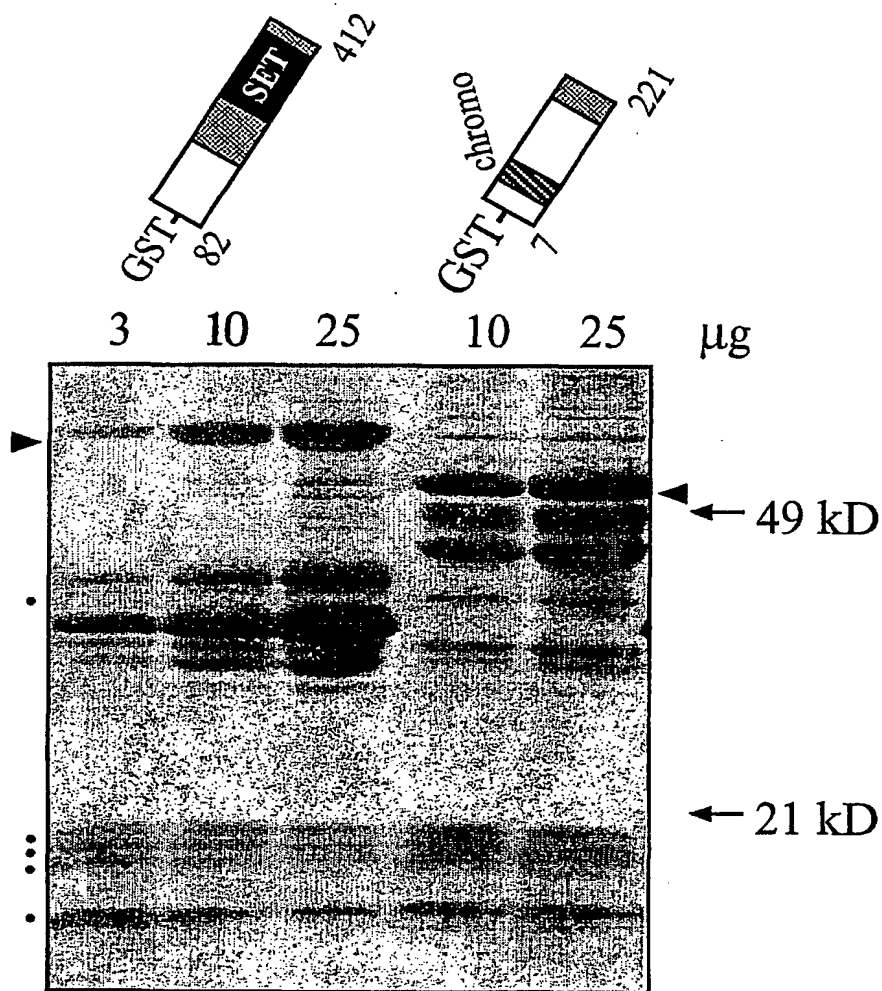


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2/33
Fig. 1/2

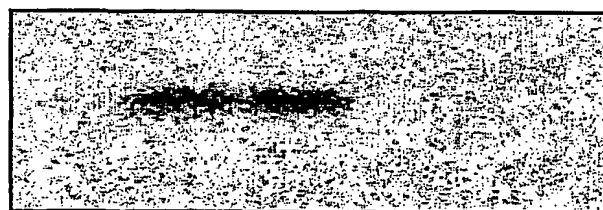
B

Suv39h1



coomassie

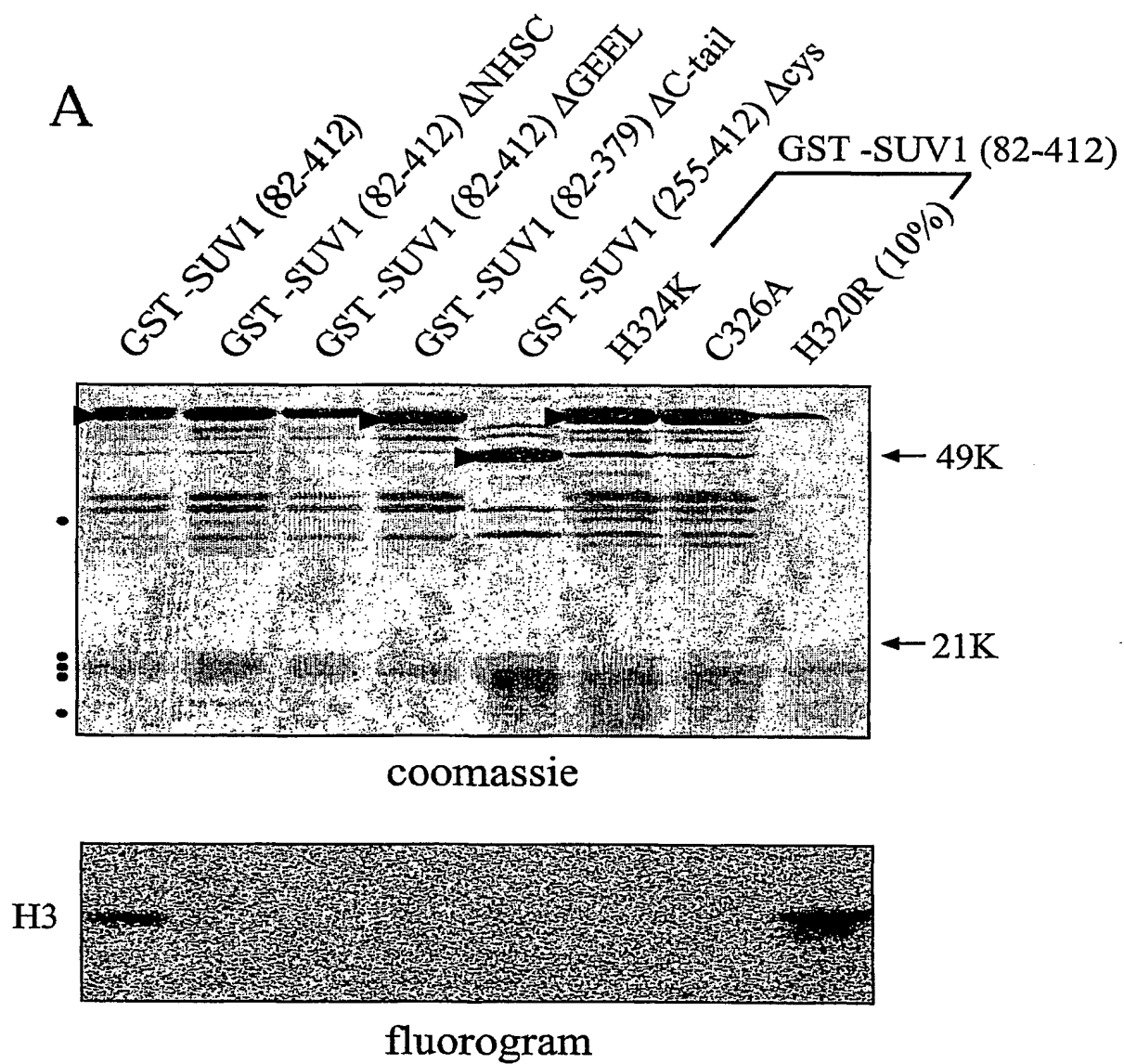
H3



fluorogram

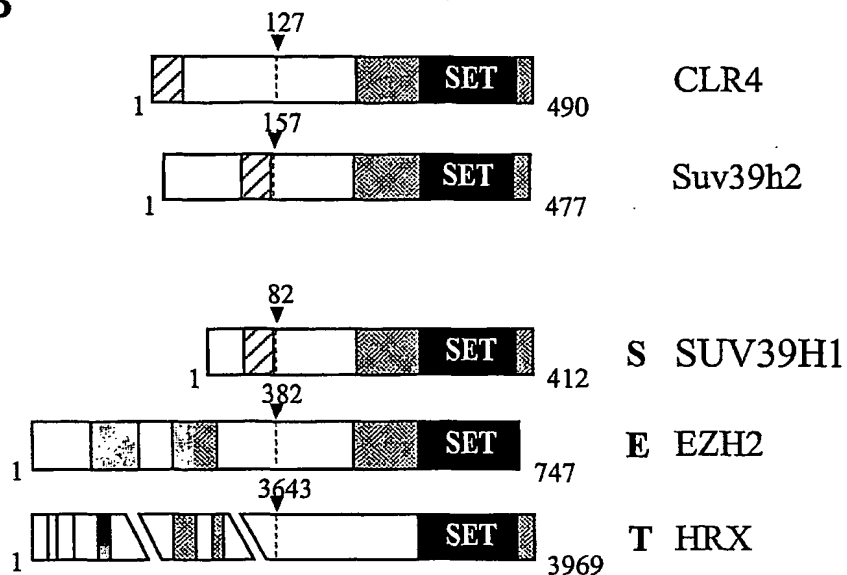
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3/33
Fig. 2A

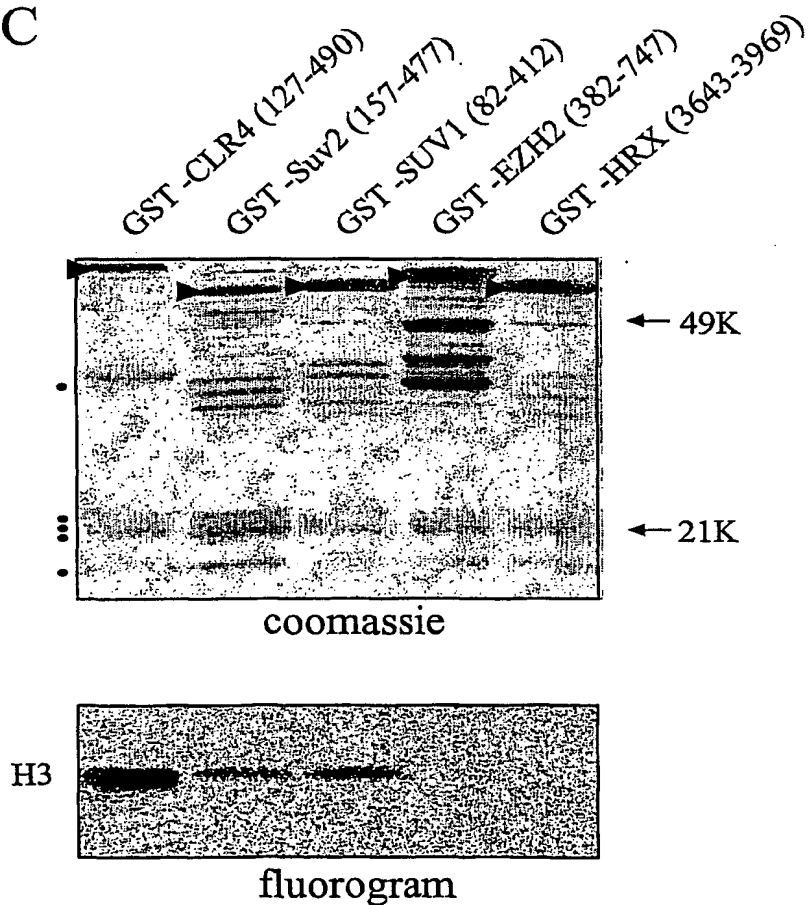


4/33
Fig. 2B

B

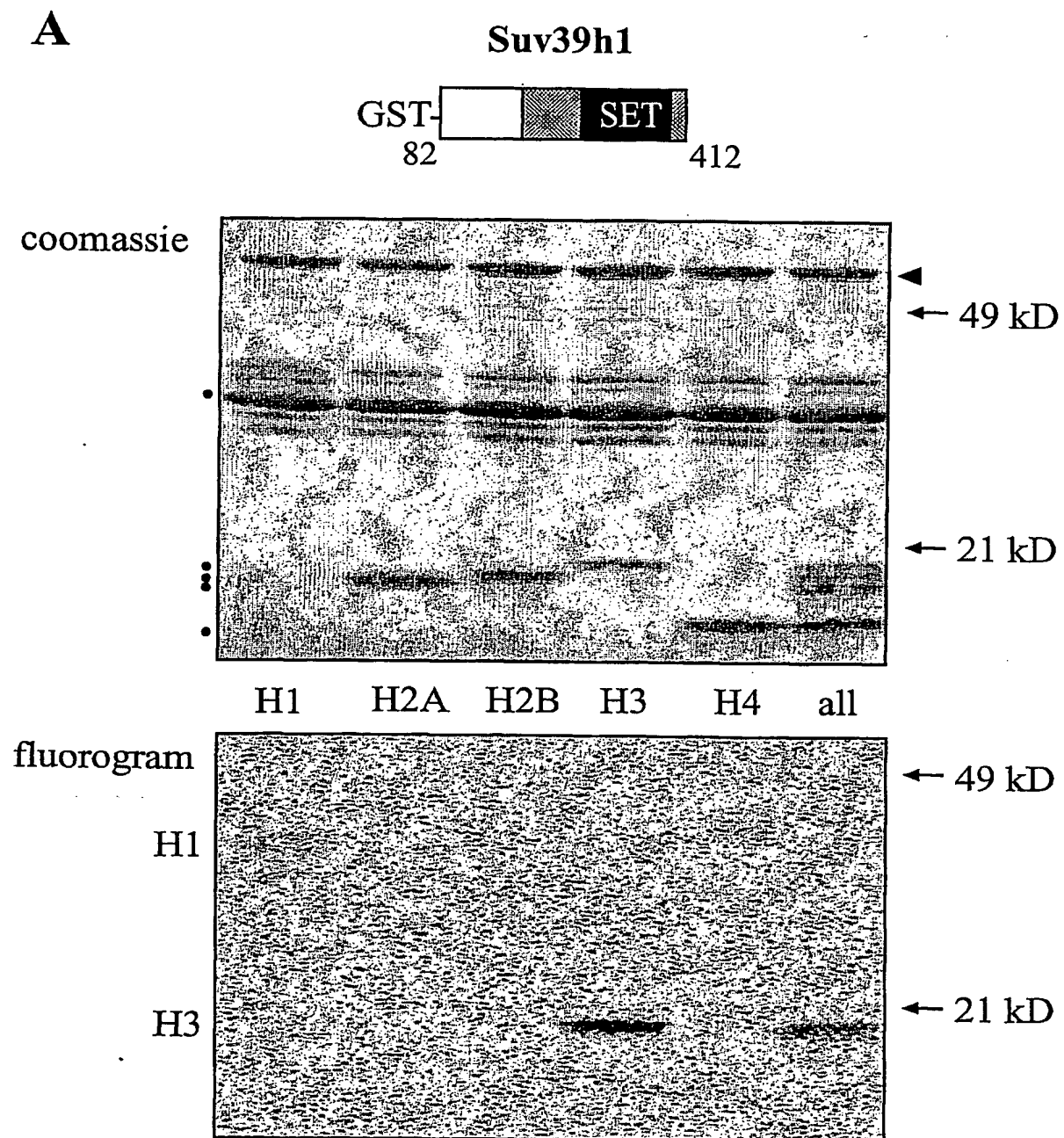


C



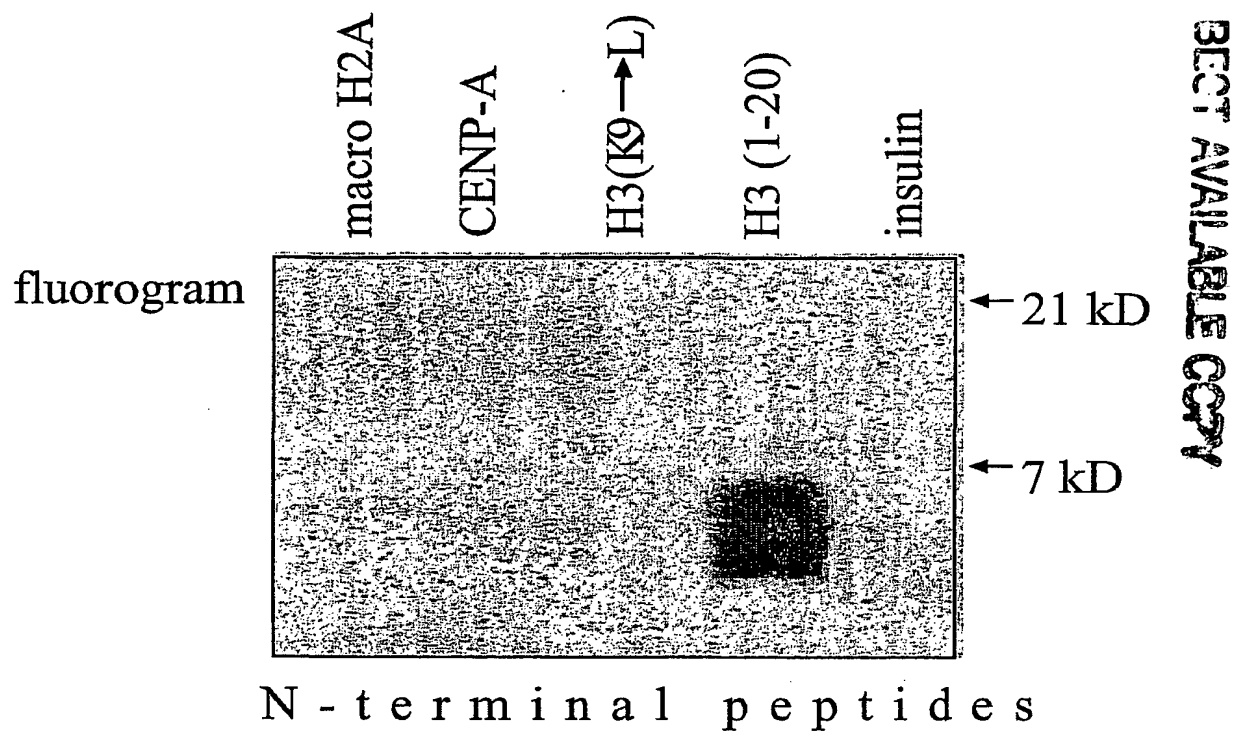
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5/33
Fig. 3A

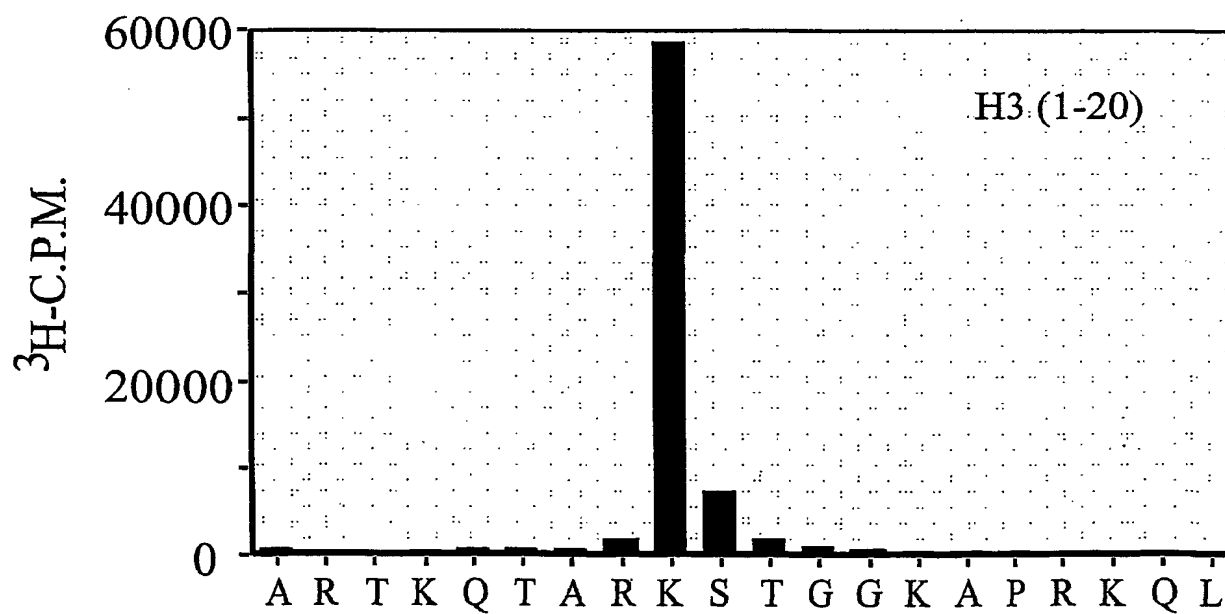


6/33
Fig. 3B

B

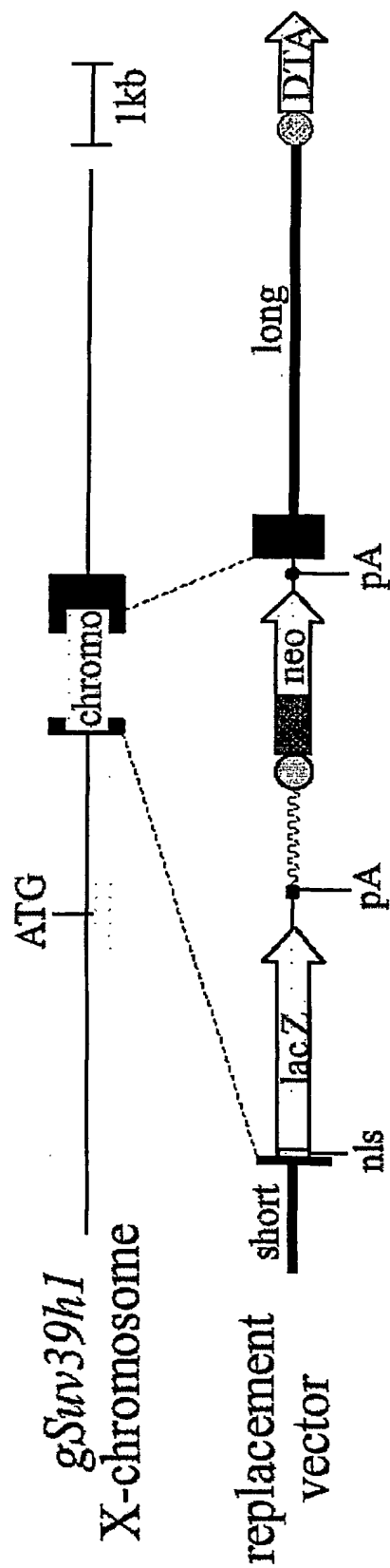


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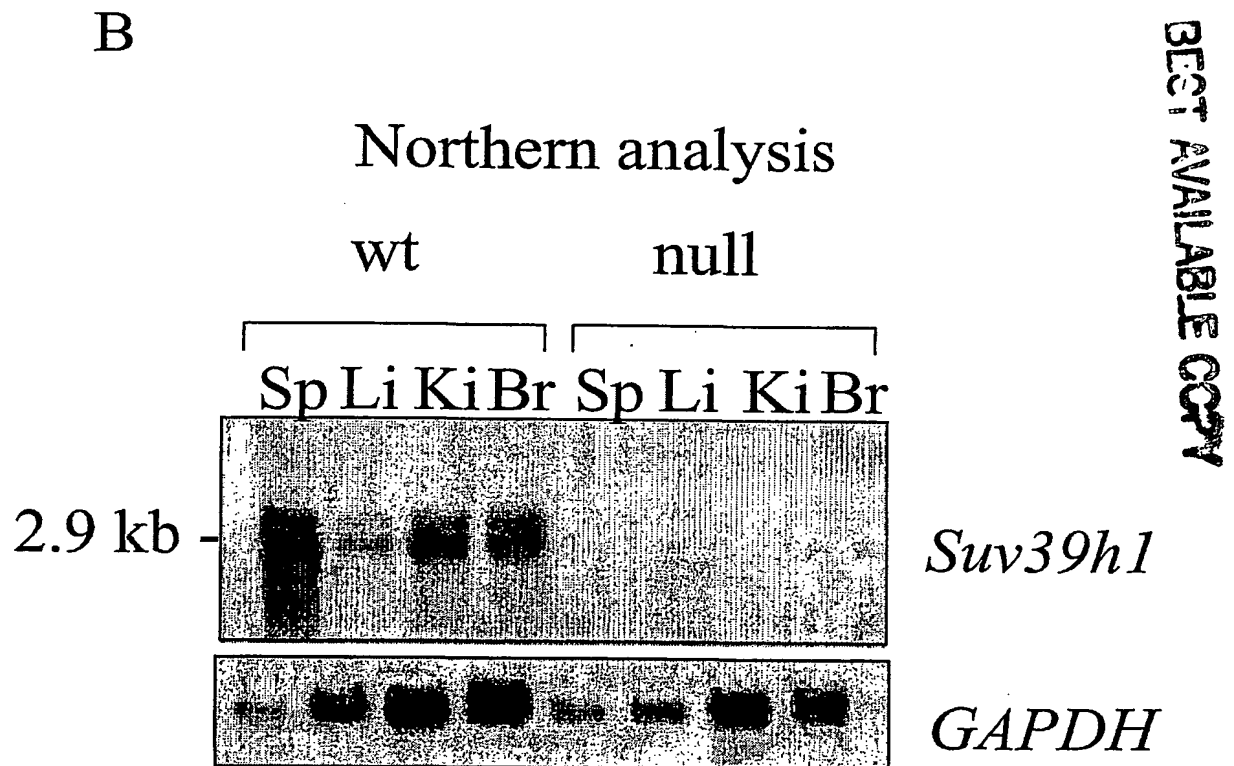


7/33
Fig. 4A

A

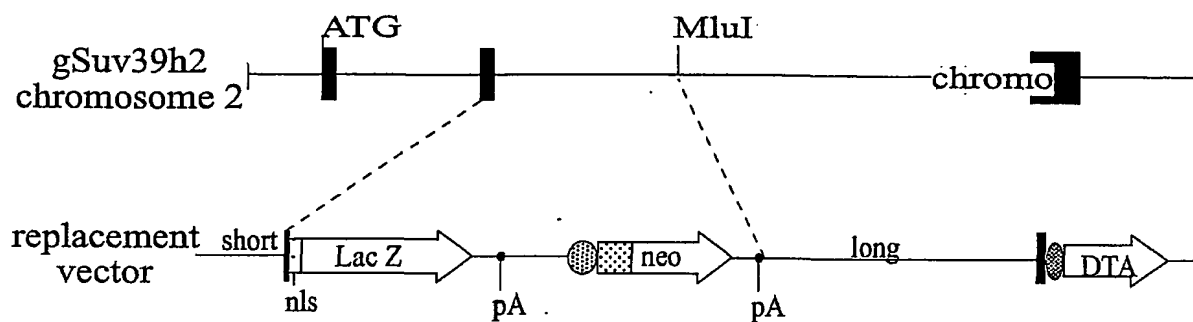


8/33
Fig. 4B

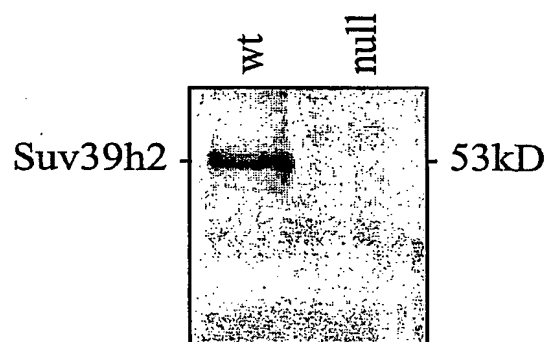


9/33
Fig. 4C

C

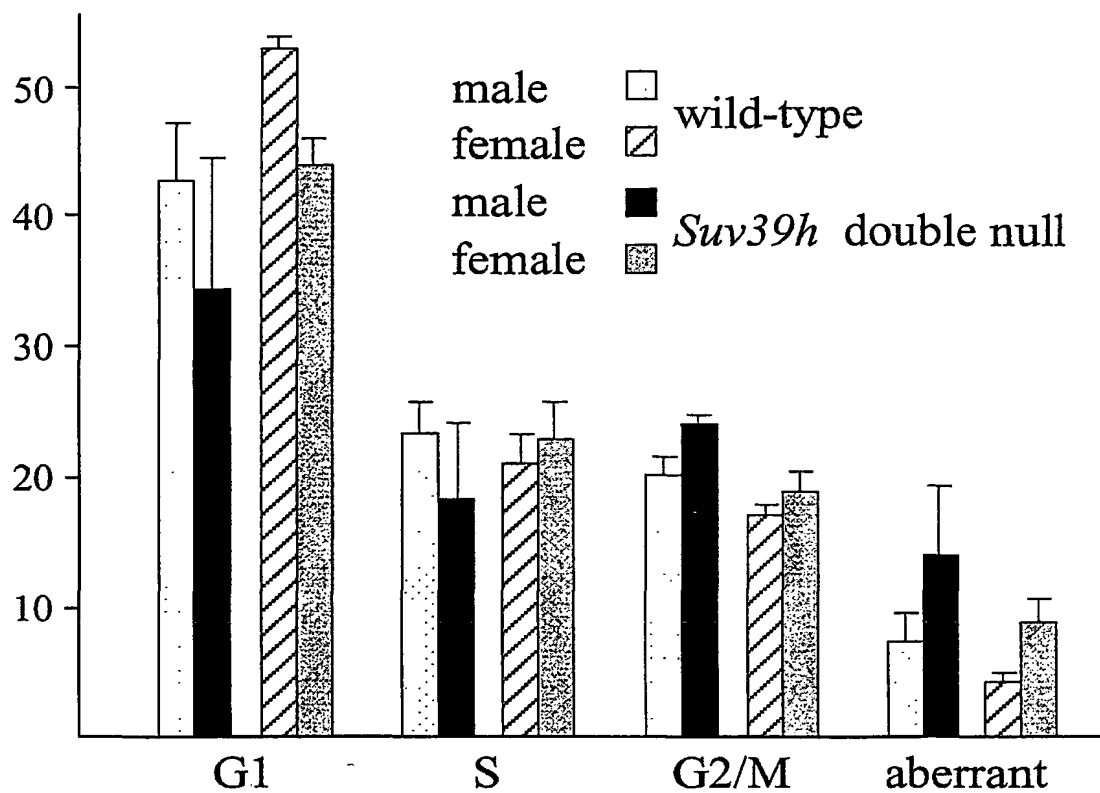


Western analysis
(testis extracts)



10/33
Fig. 5

A



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B



11/33

Fig. 6

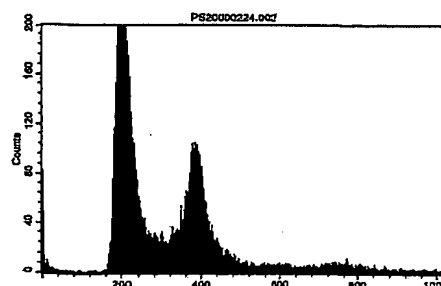
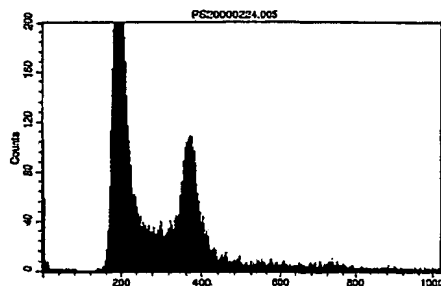
A

Passage 3

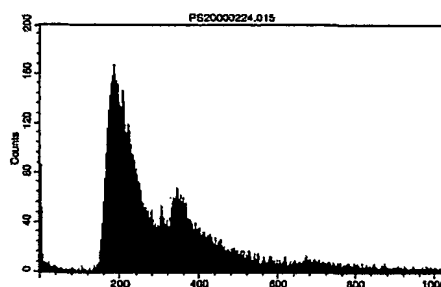
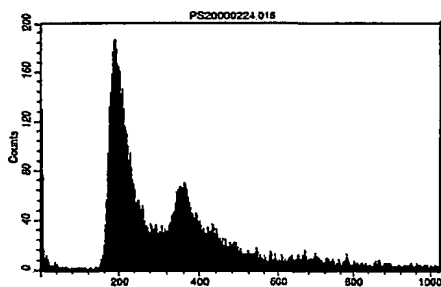
Female

Male

wt



double
null



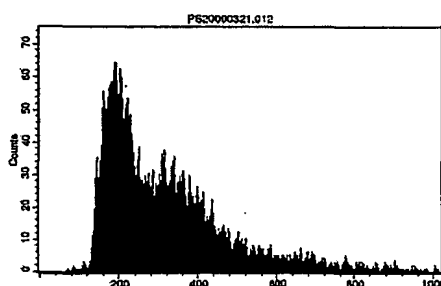
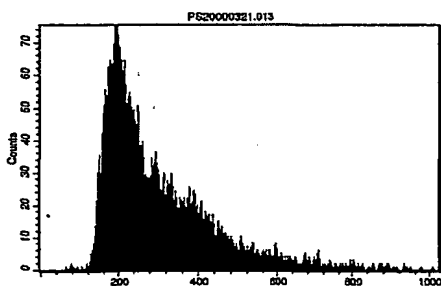
2N 4N

2N 4N

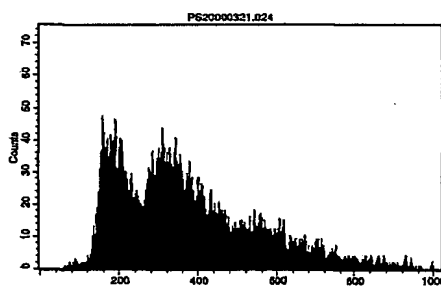
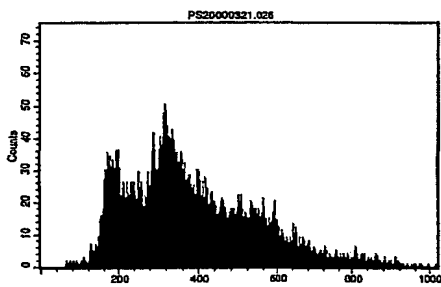
B

Passage 8

wt



double
null



2N 4N 8N

2N 4N 8N

DNA content

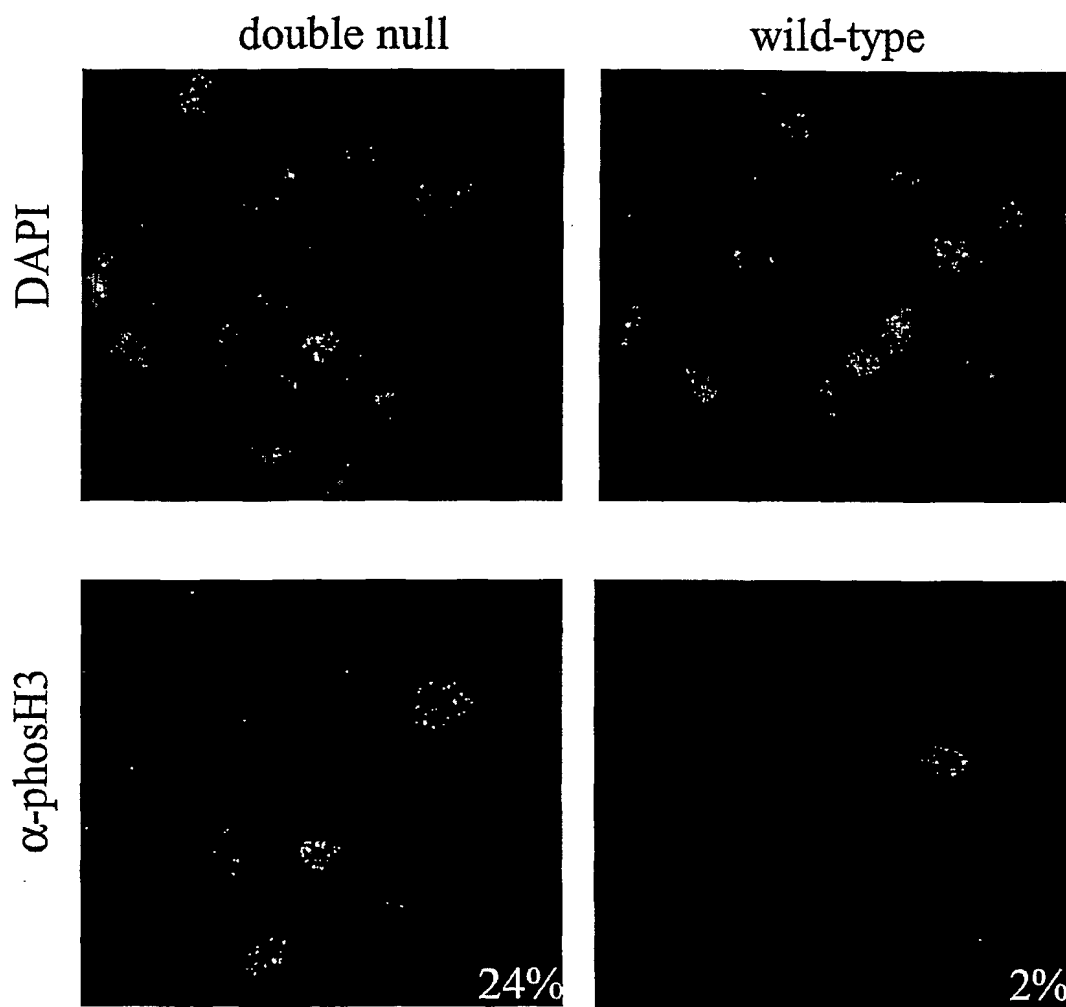


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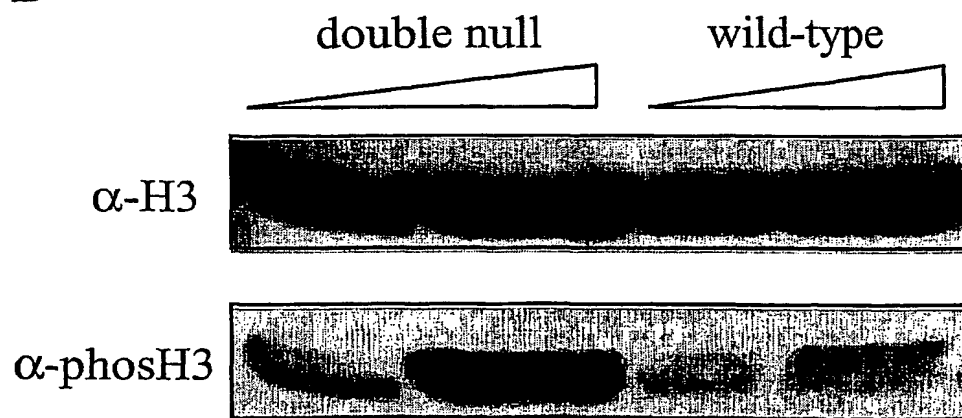
12/33

Fig. 7

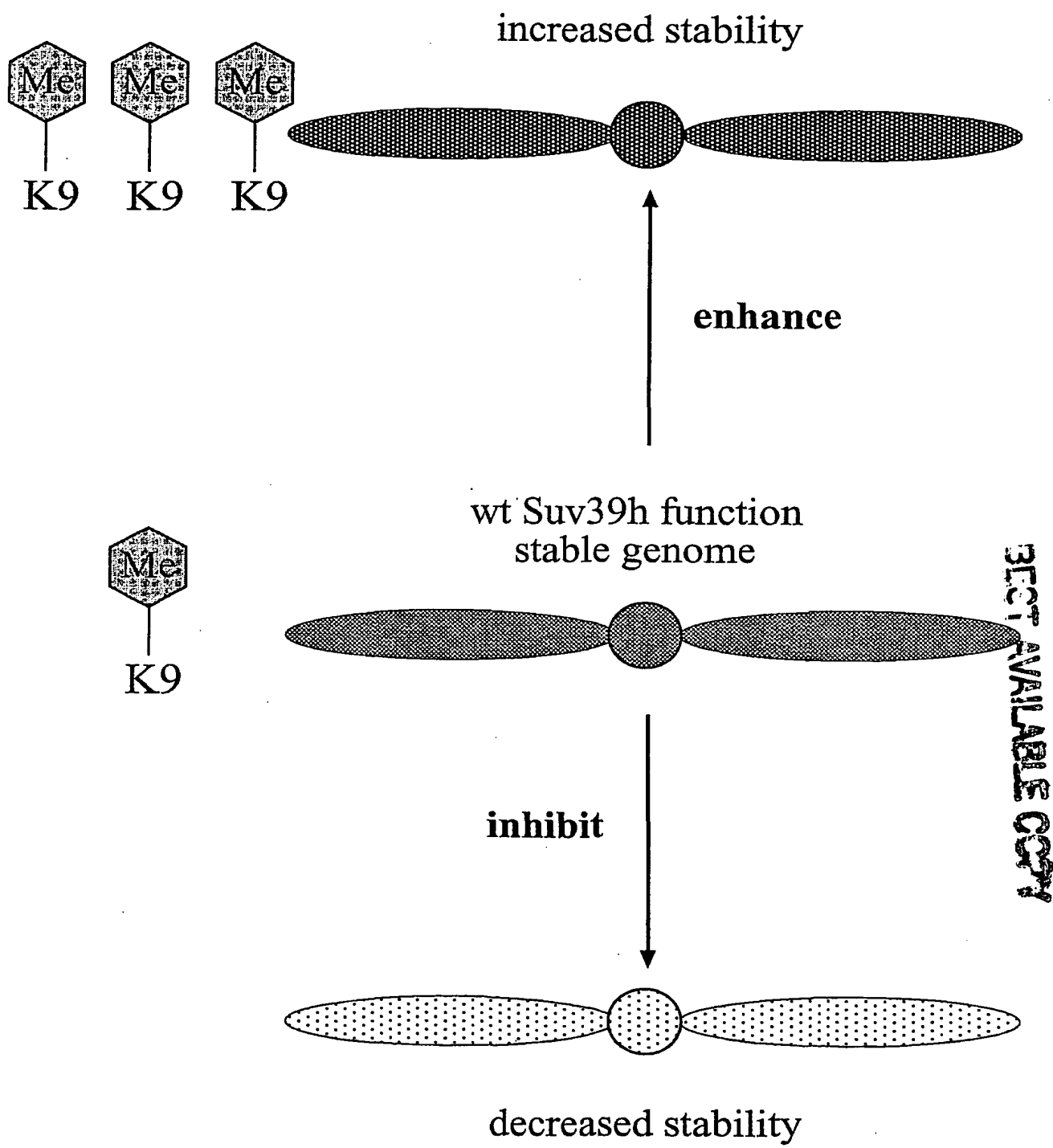
A



B

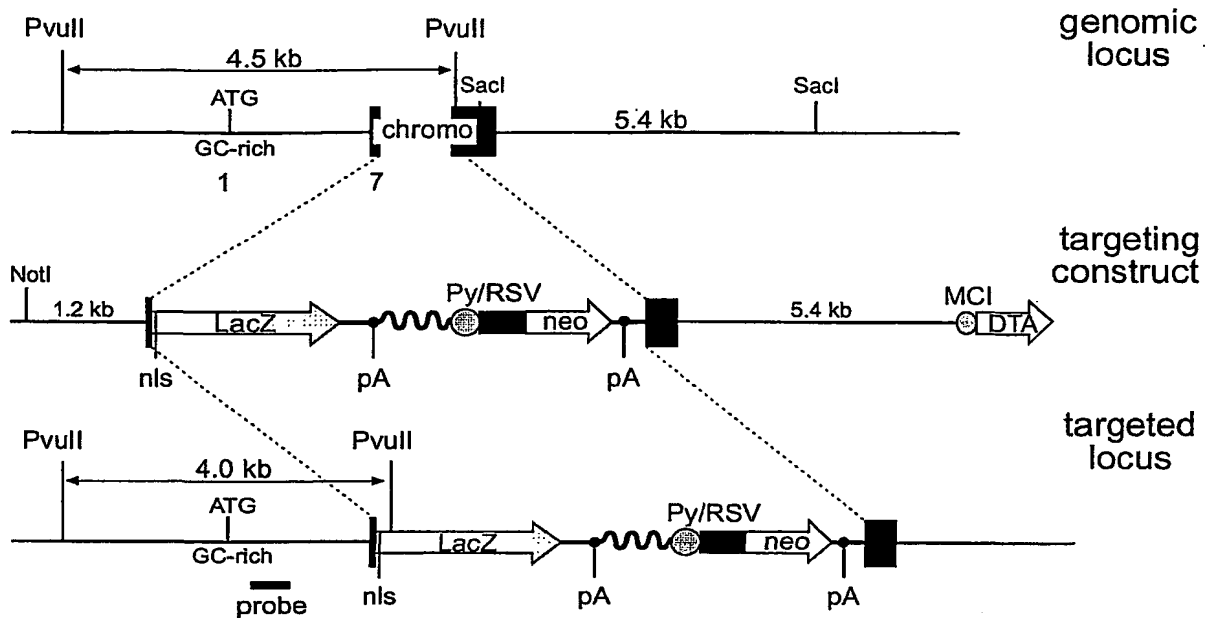


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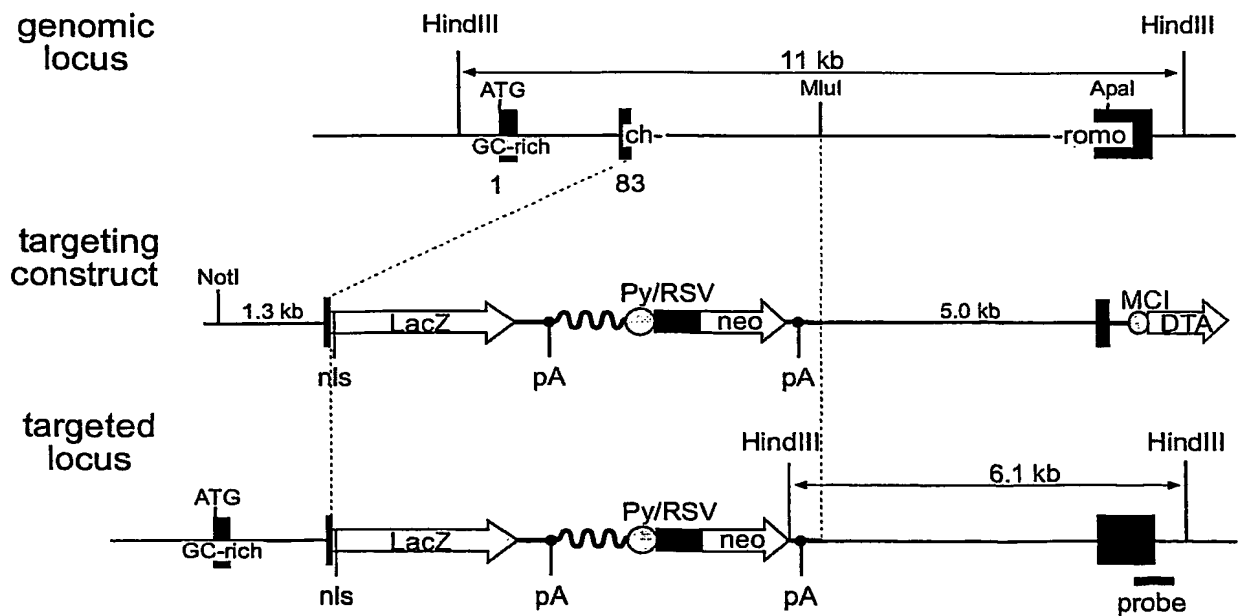
13/33
Fig. 8

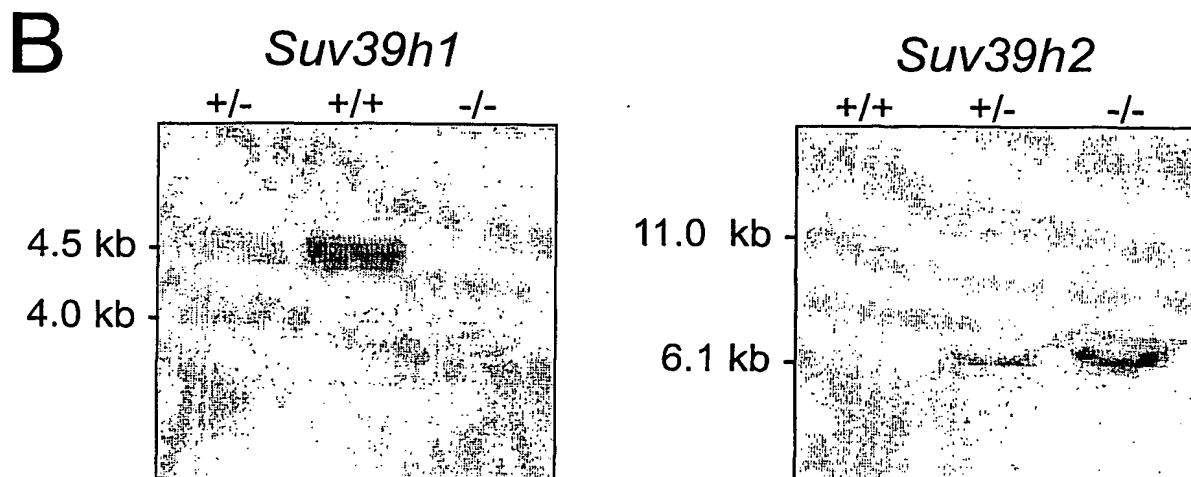
14/33
Fig. 9A

A *Suv39h1* (X chromosome)



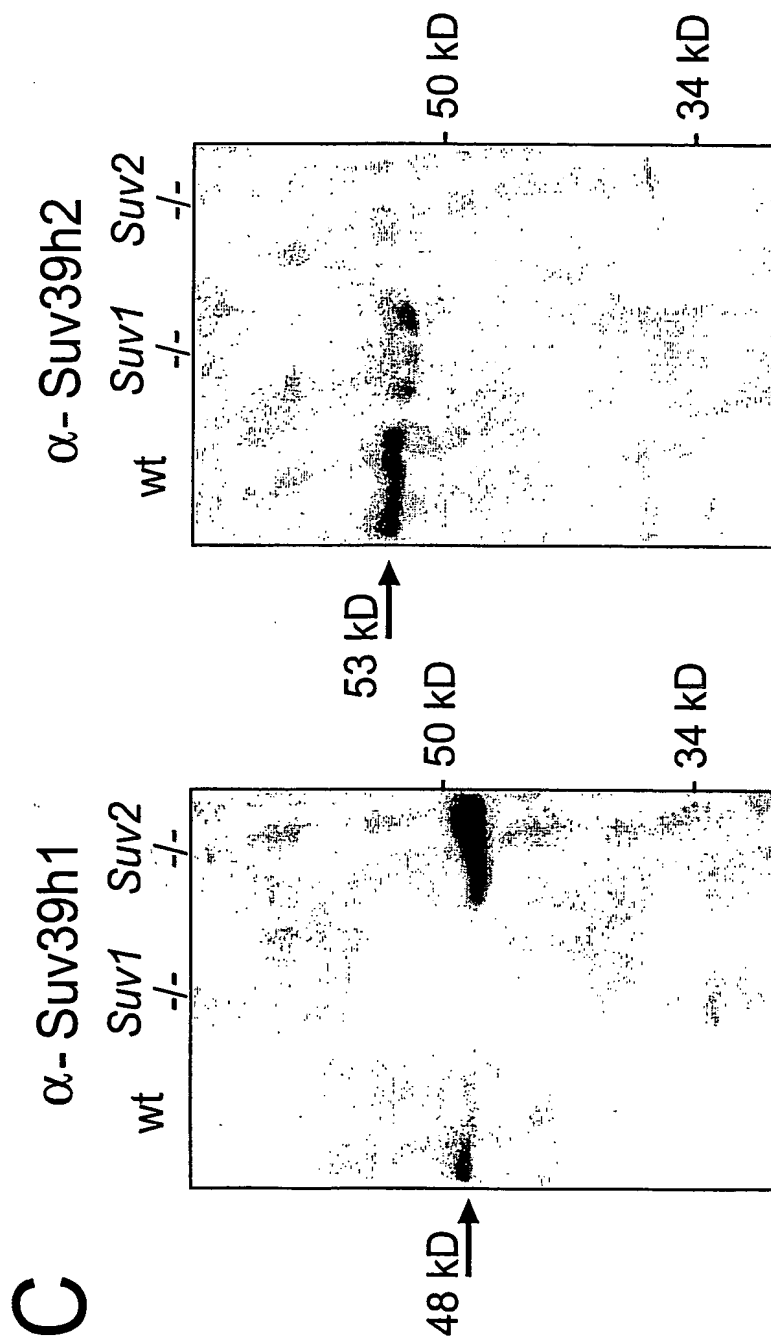
Suv39h2 (chromosome 2)



15/33**Fig. 9B****BEST AVAILABLE COPY**

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Fig. 9C



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17/33

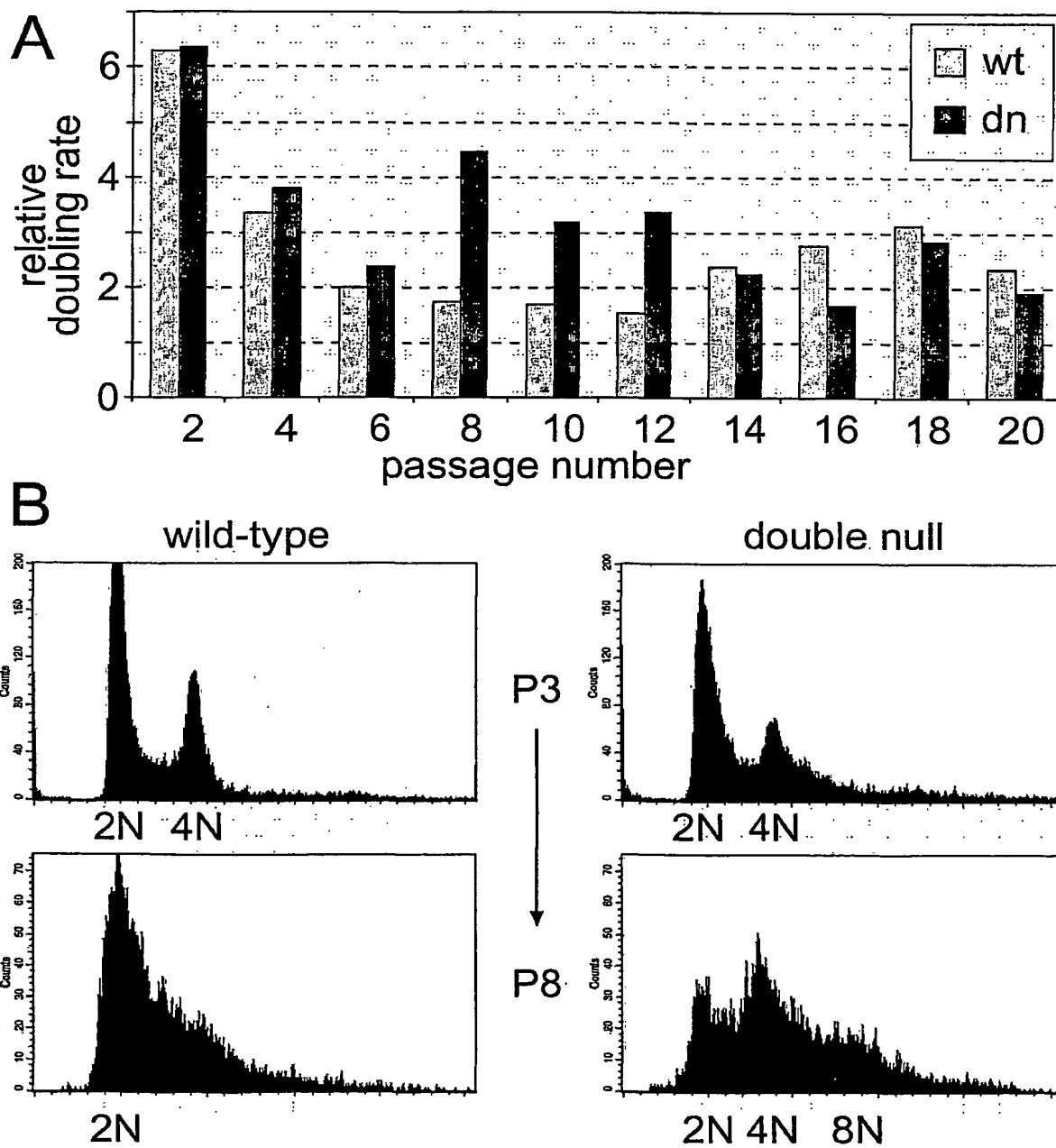
Fig. 9D

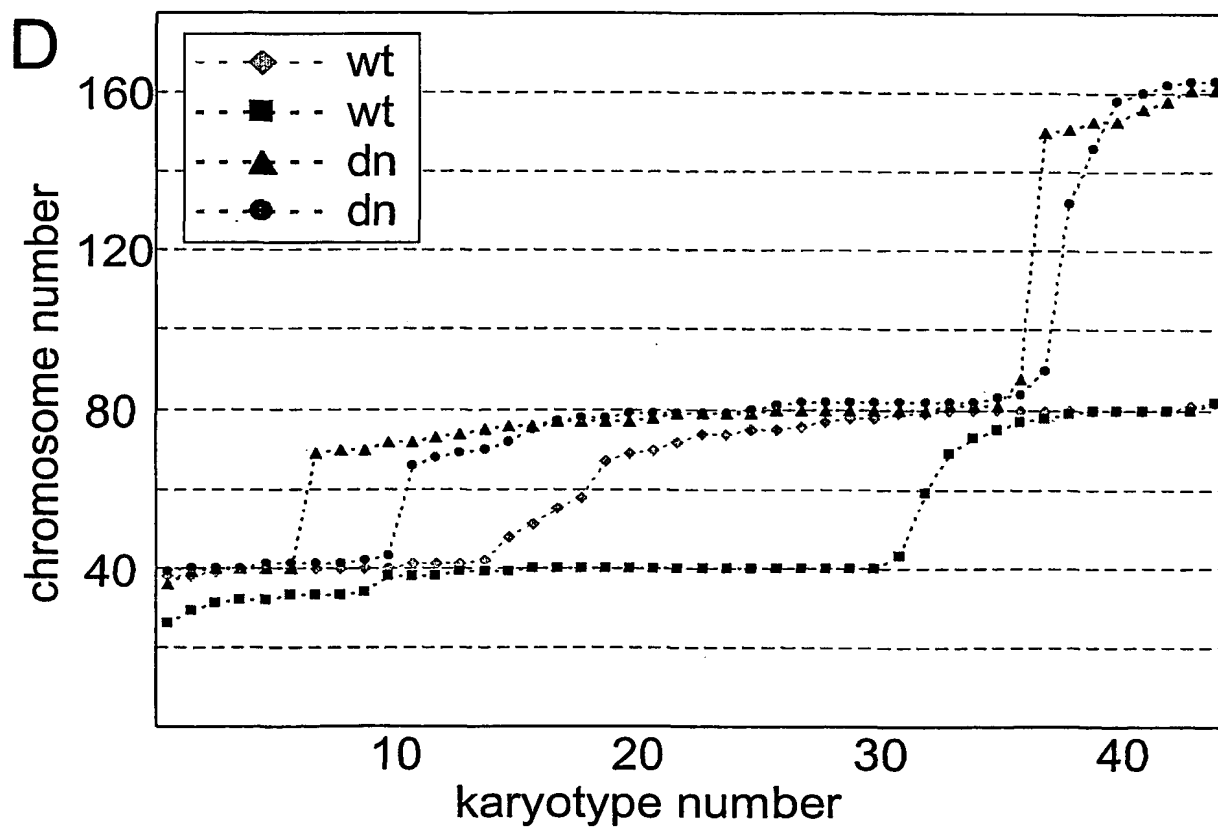
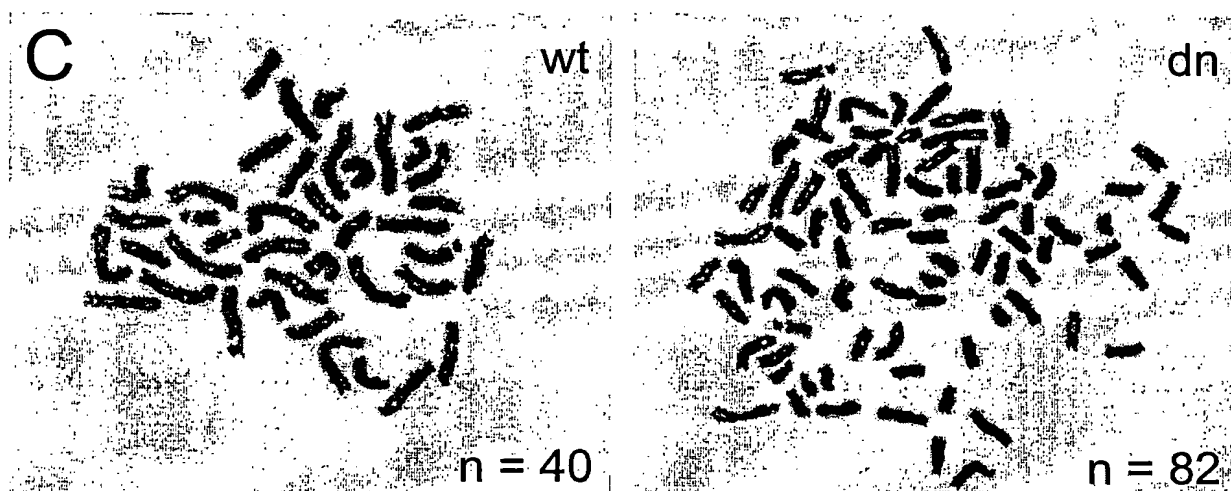
D



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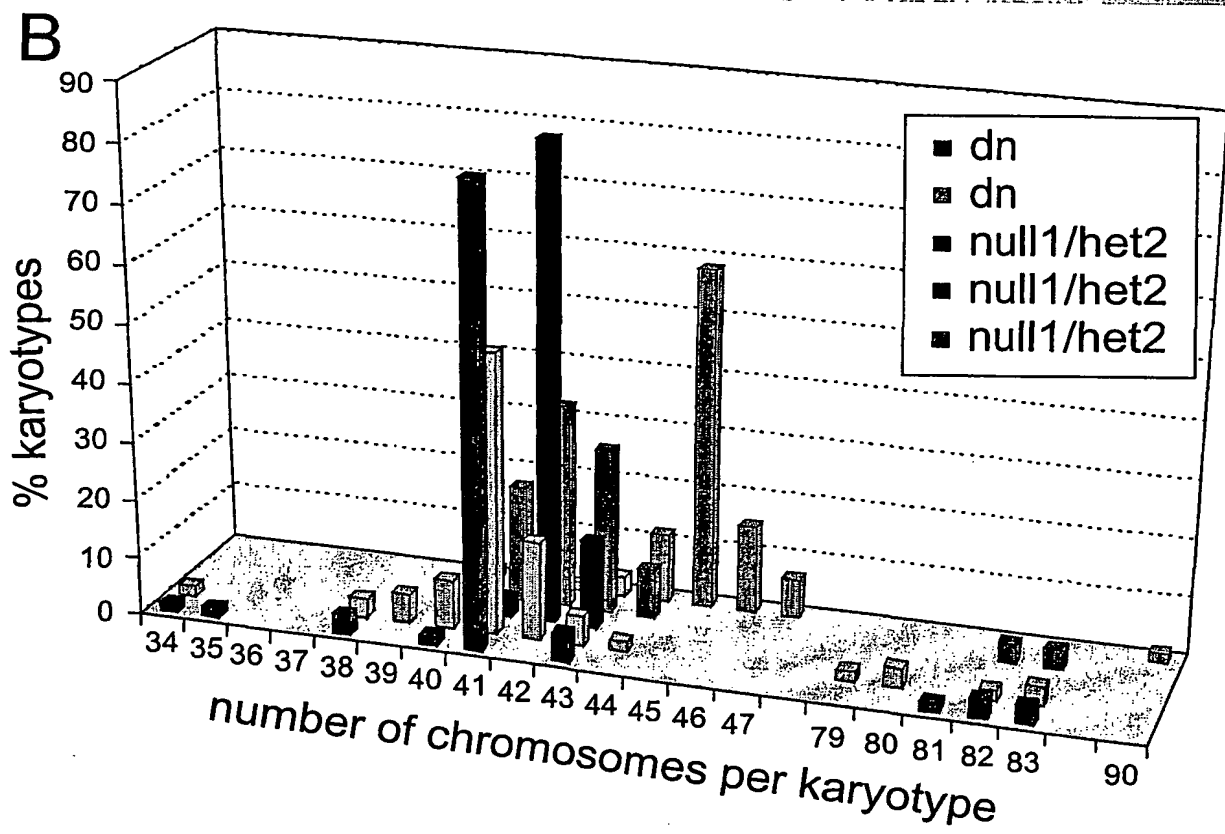
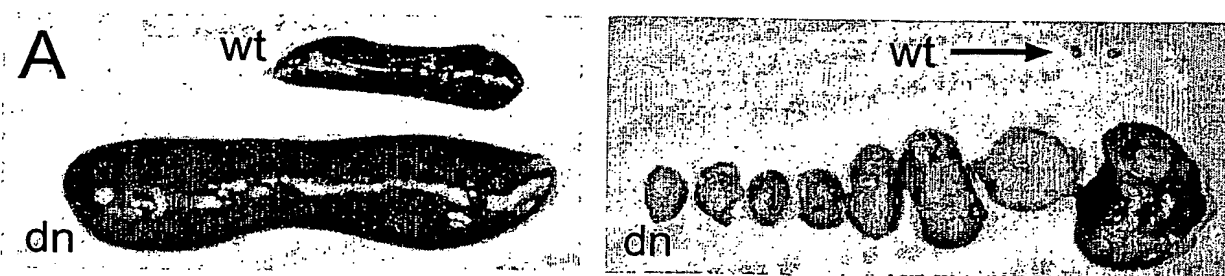
18/33
Fig. 10A,B



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Fig. 10C,D

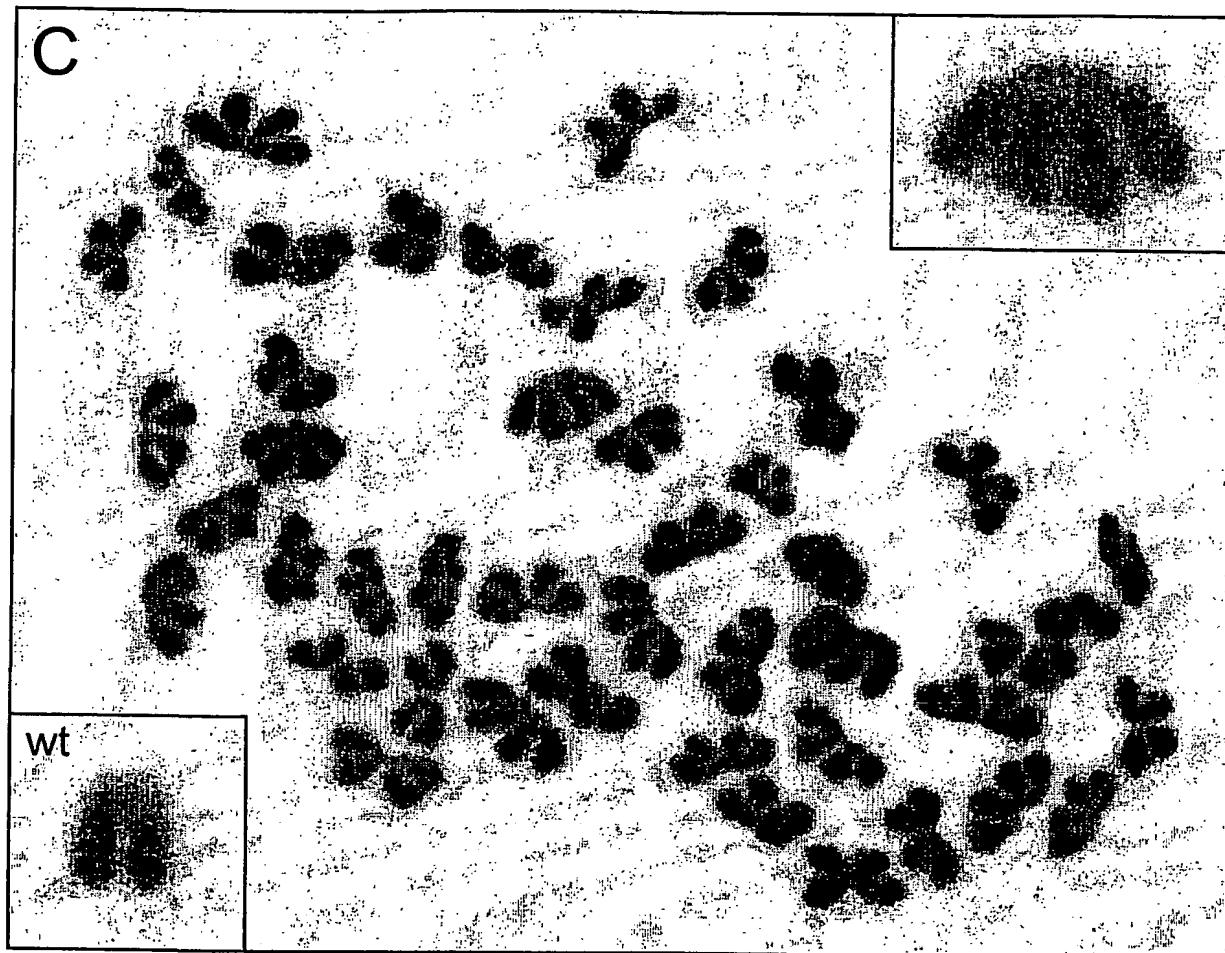
20/33

Fig. 11A,B



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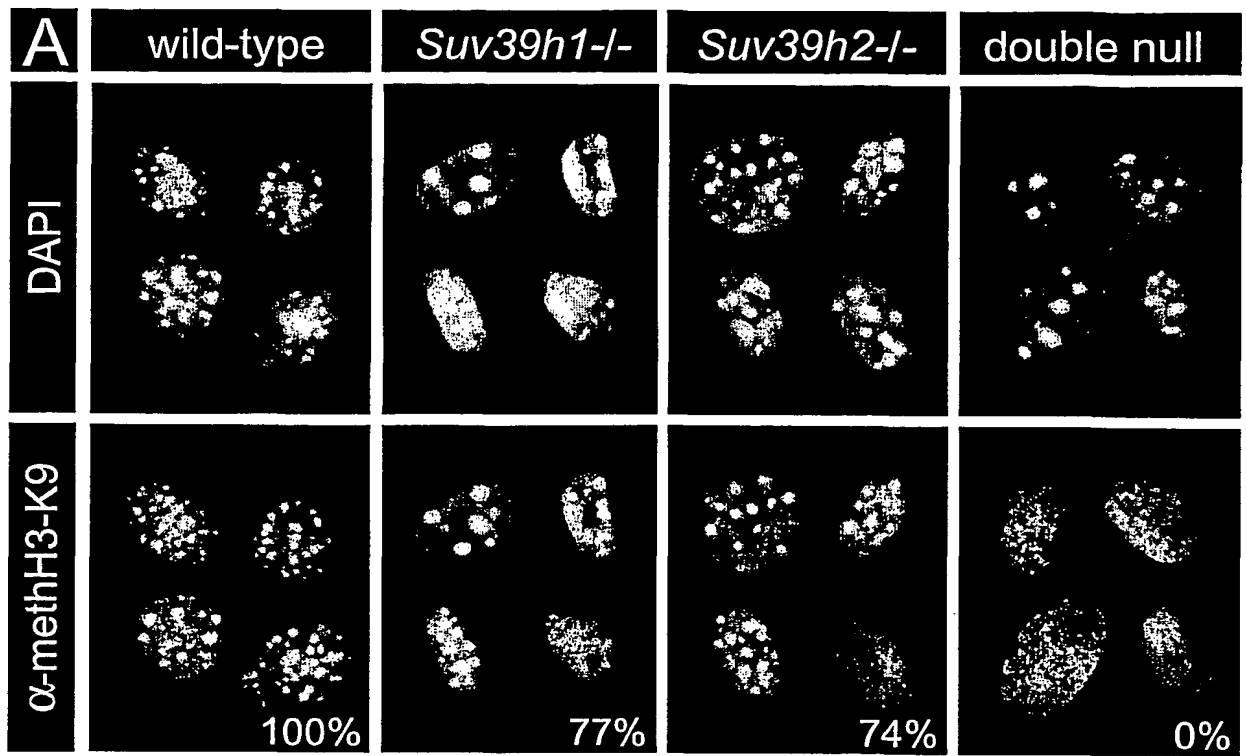
Fig. 11C



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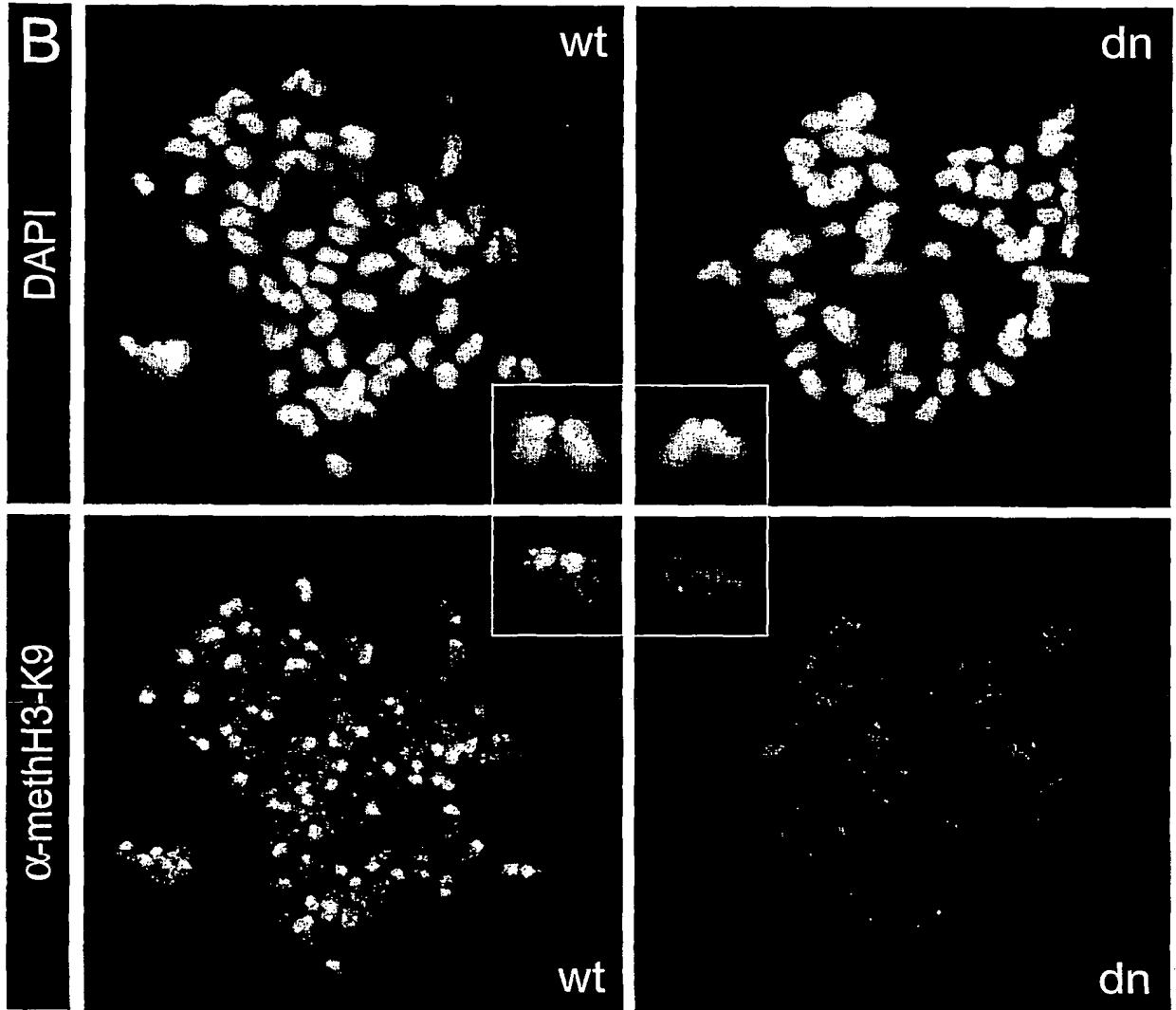
22/33

Fig. 12A



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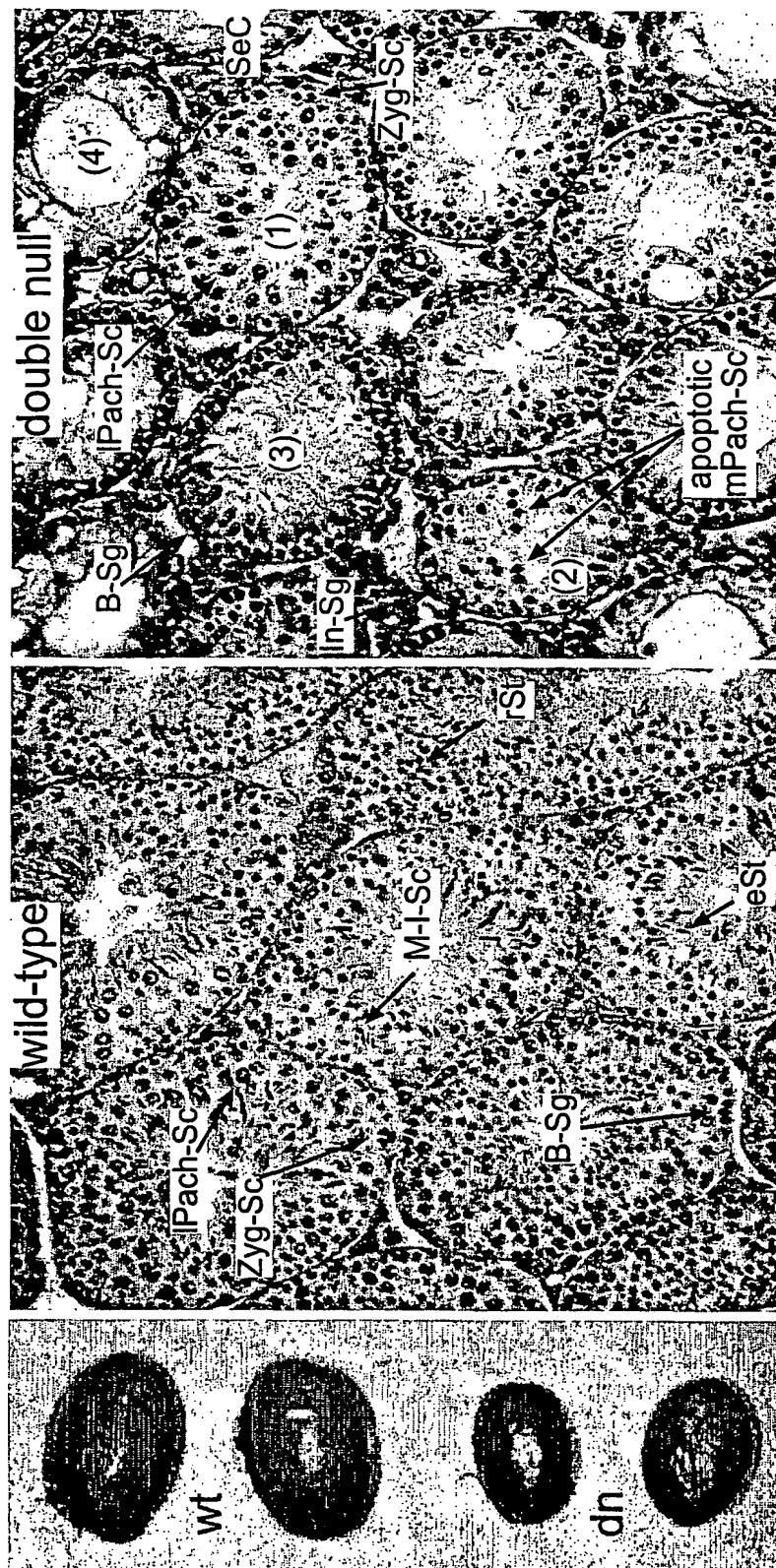
Fig. 12B



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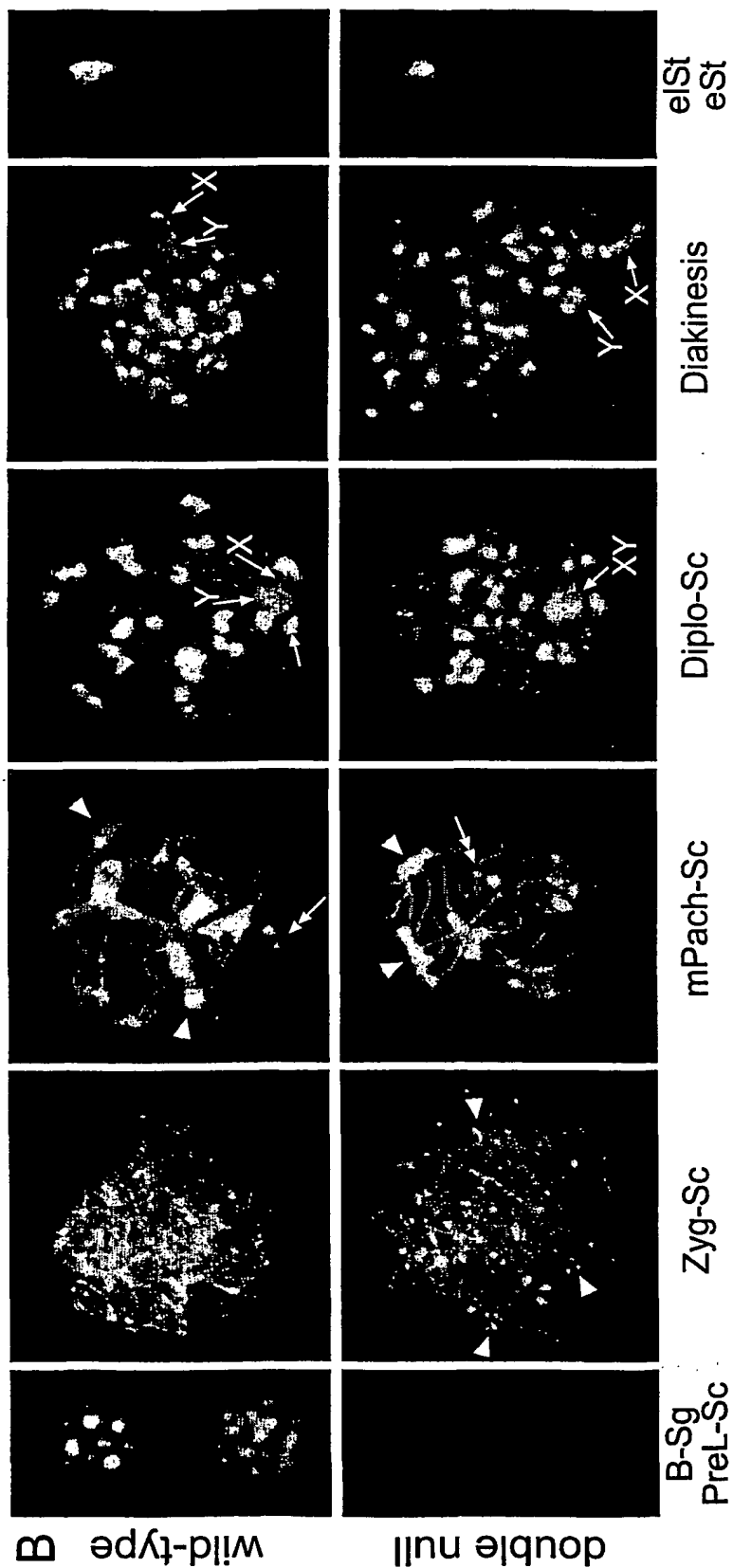
Fig. 13A



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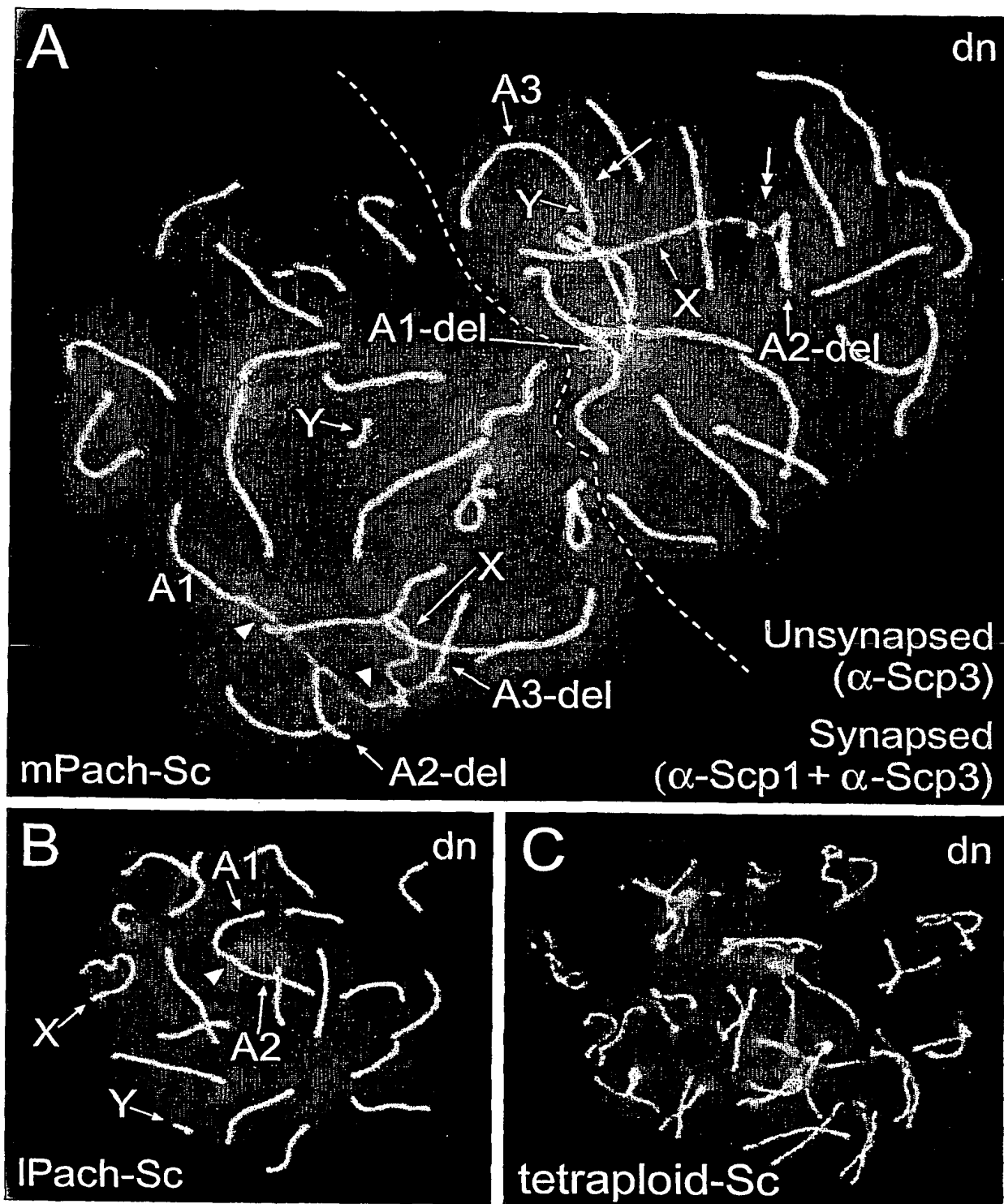
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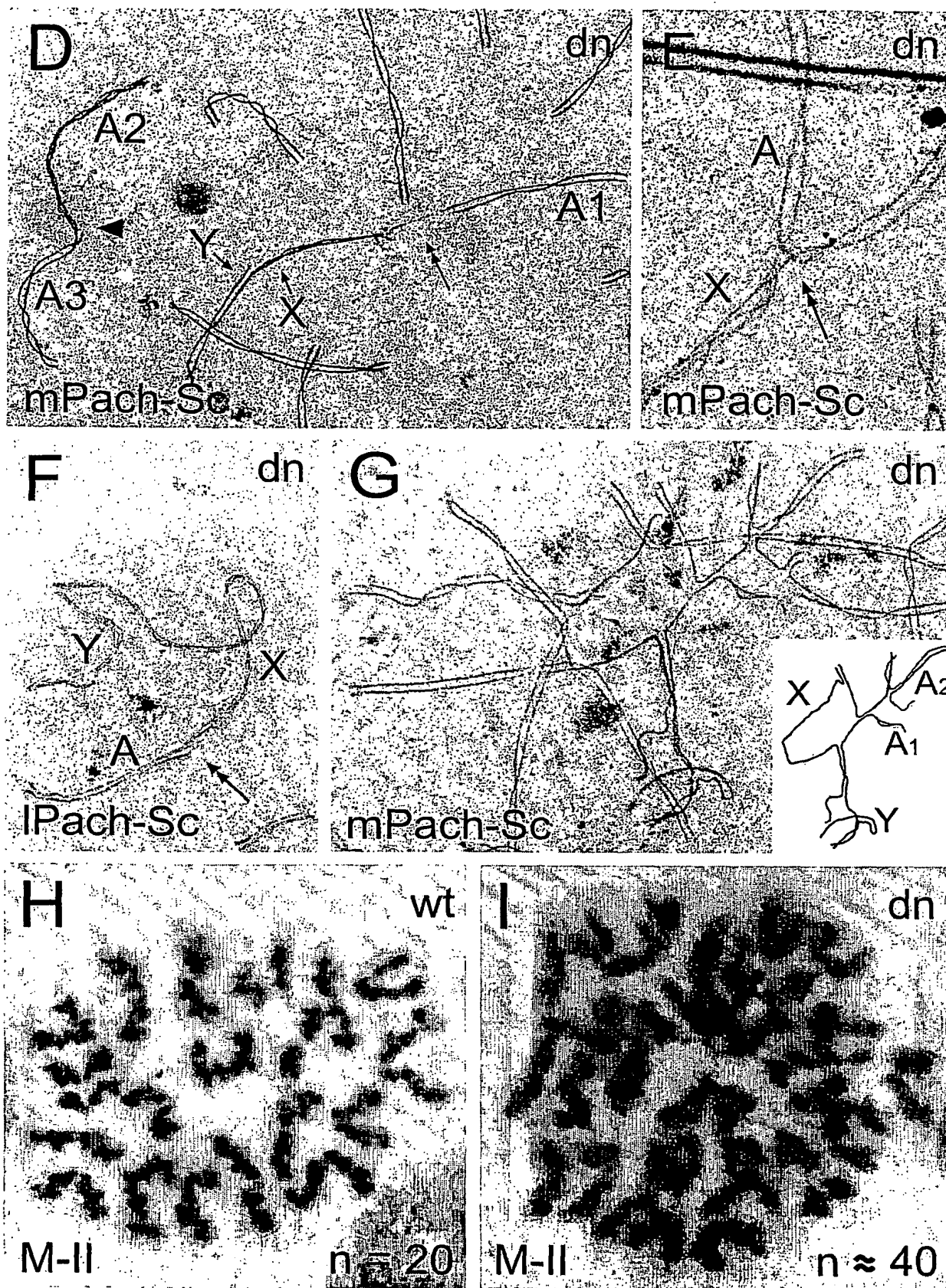
25/33
Fig. 13B



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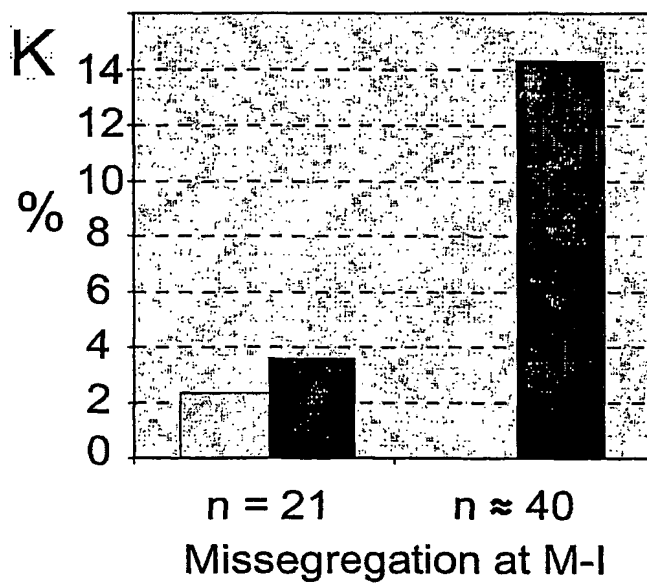
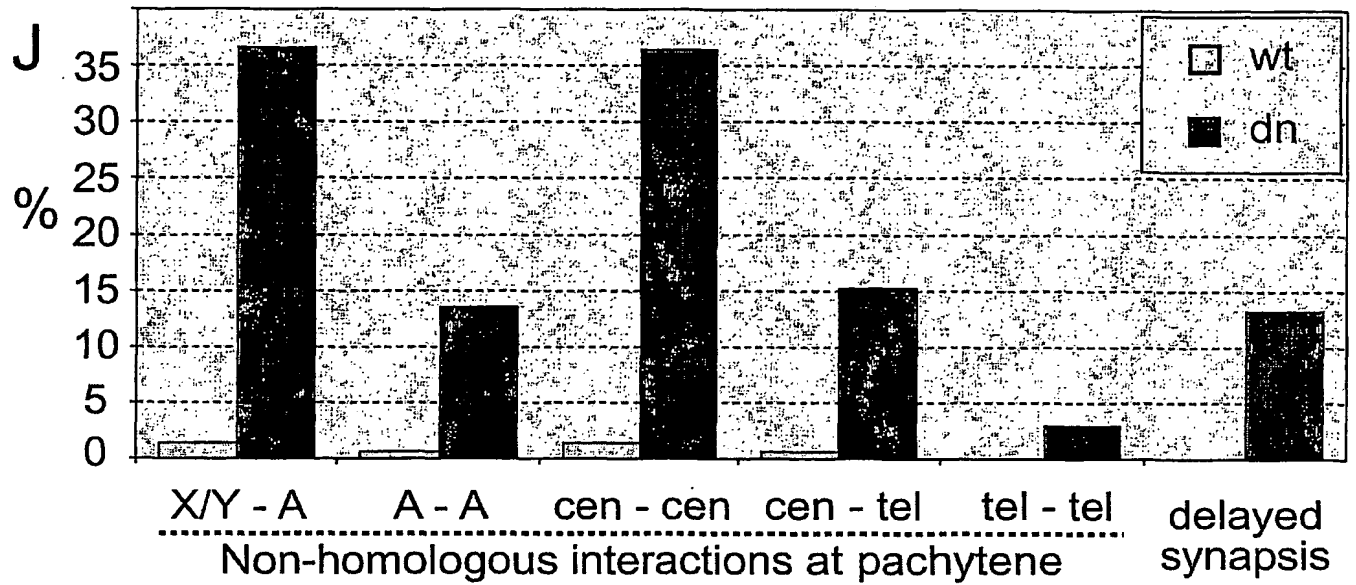
26/33
Fig. 14A-C



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Fig. 14D-I

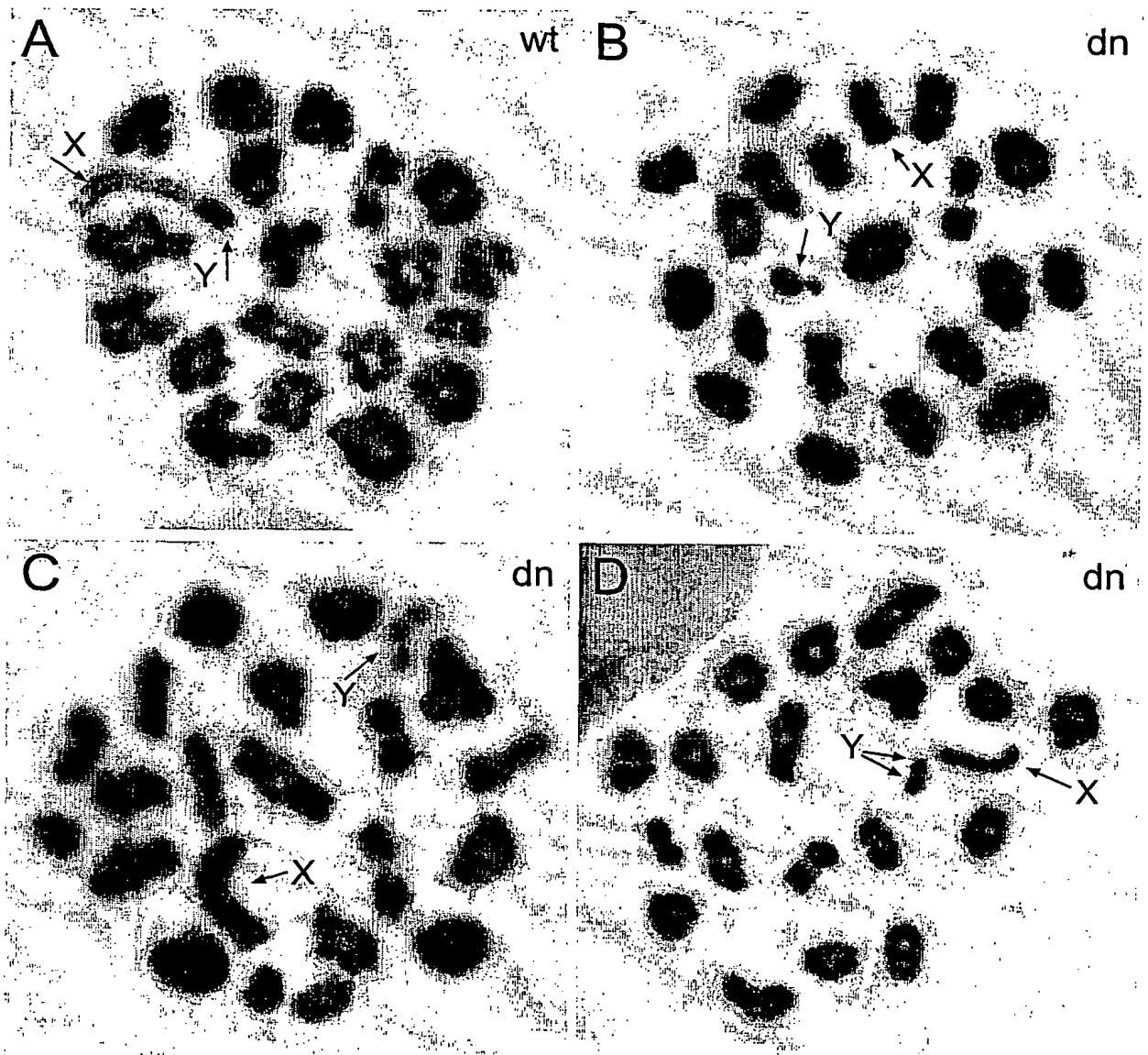
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28/33
Fig. 14J,K



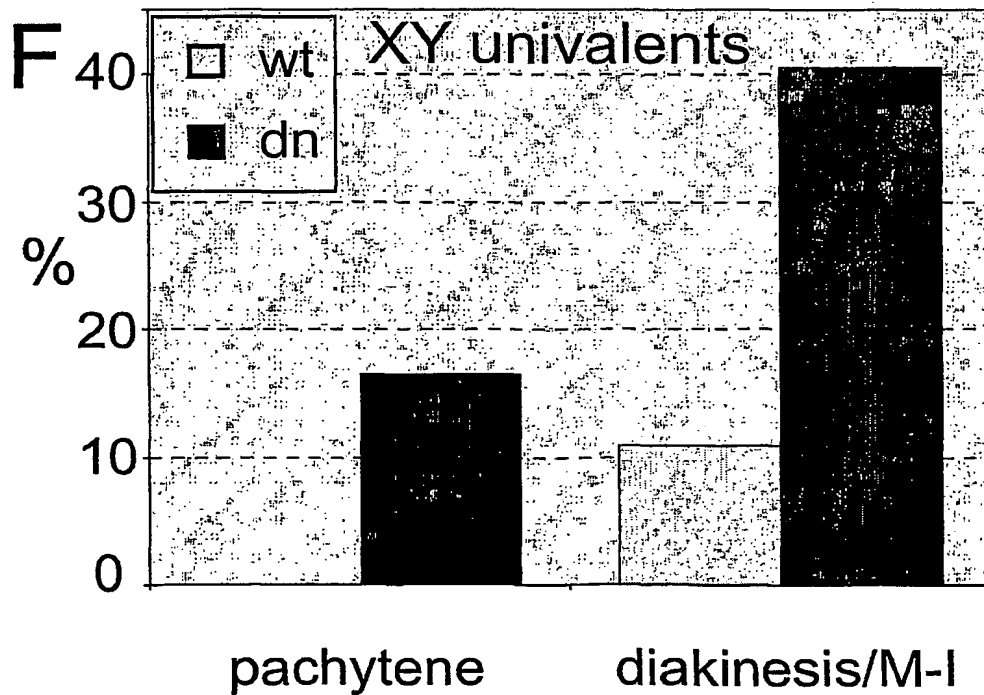
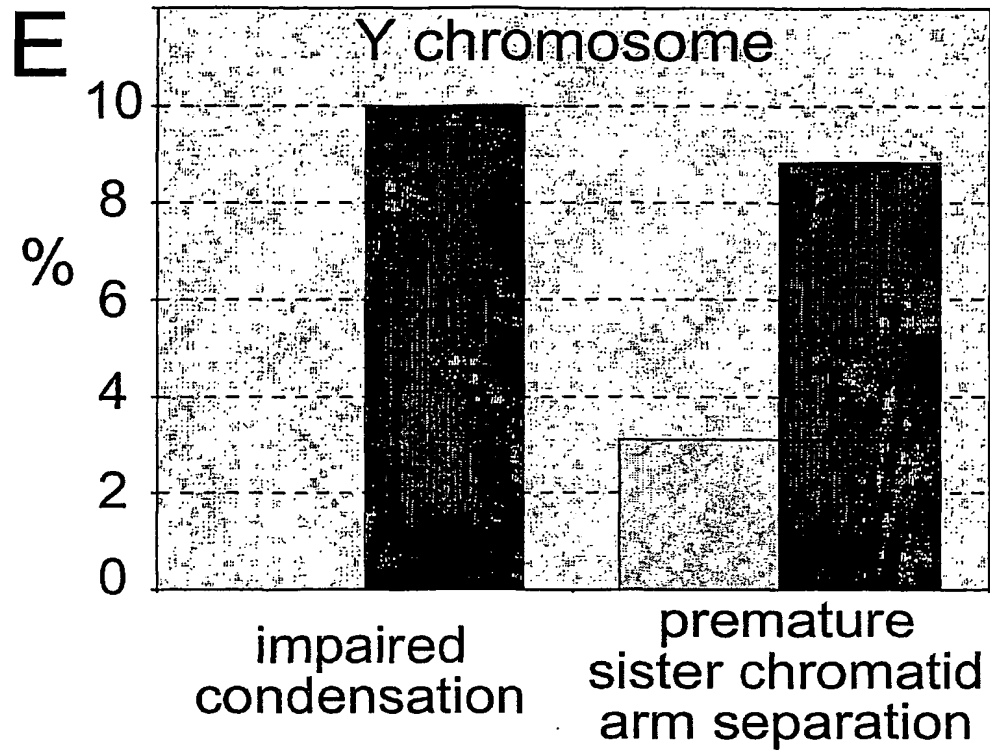
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29/33
Fig. 15A-D



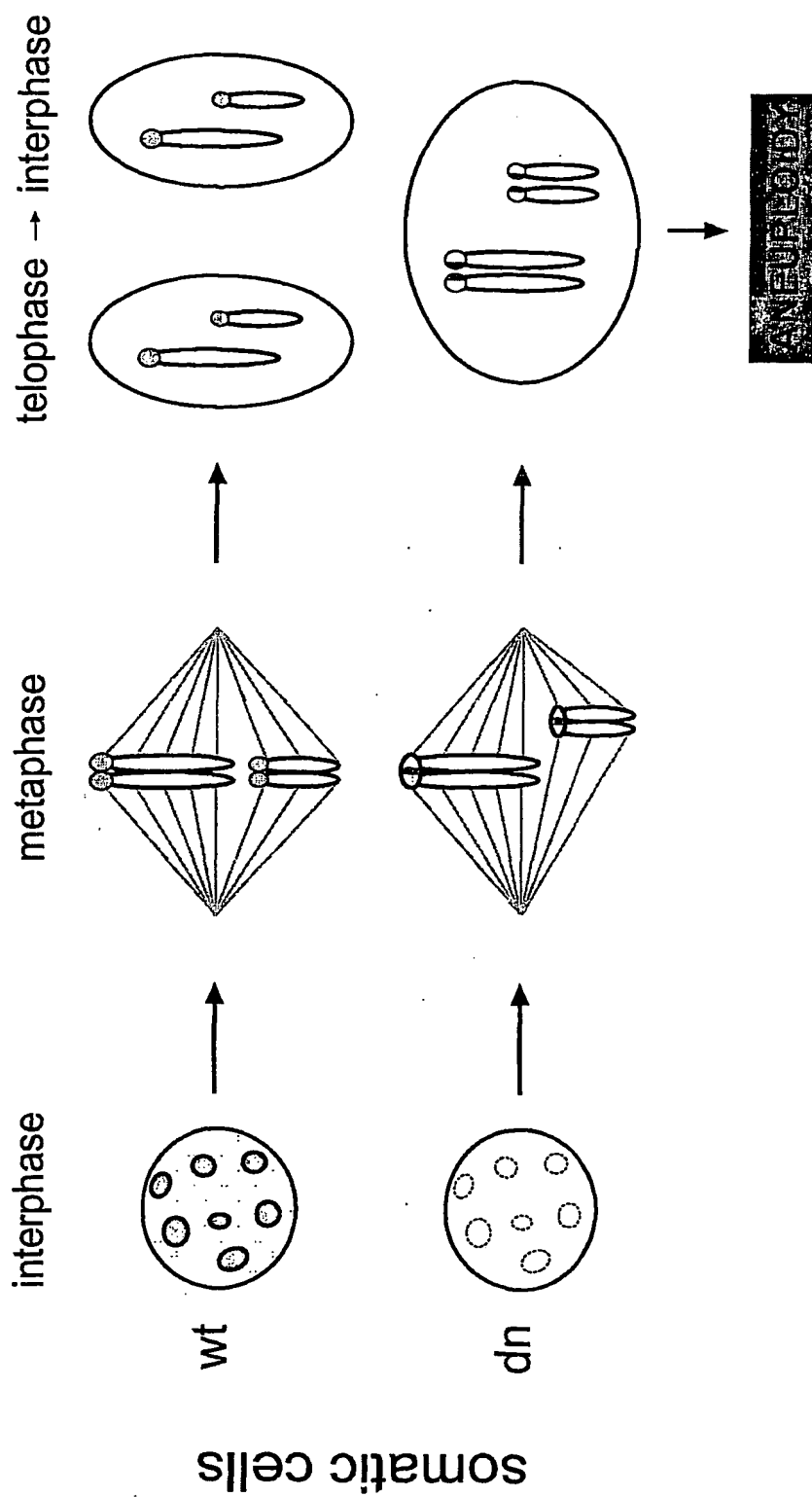
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30/33
Fig. 15E,F



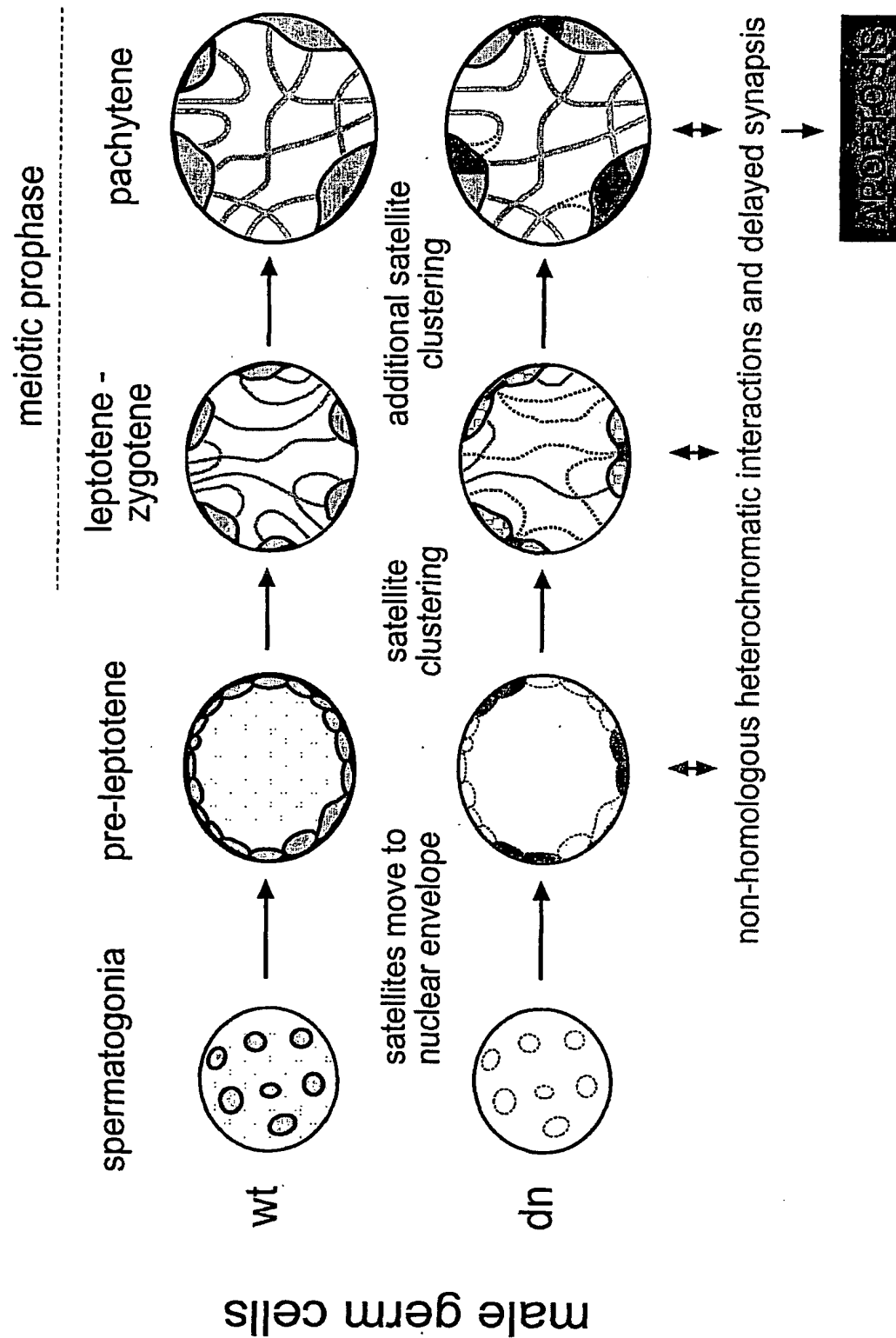
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31/33
Fig. 16/1

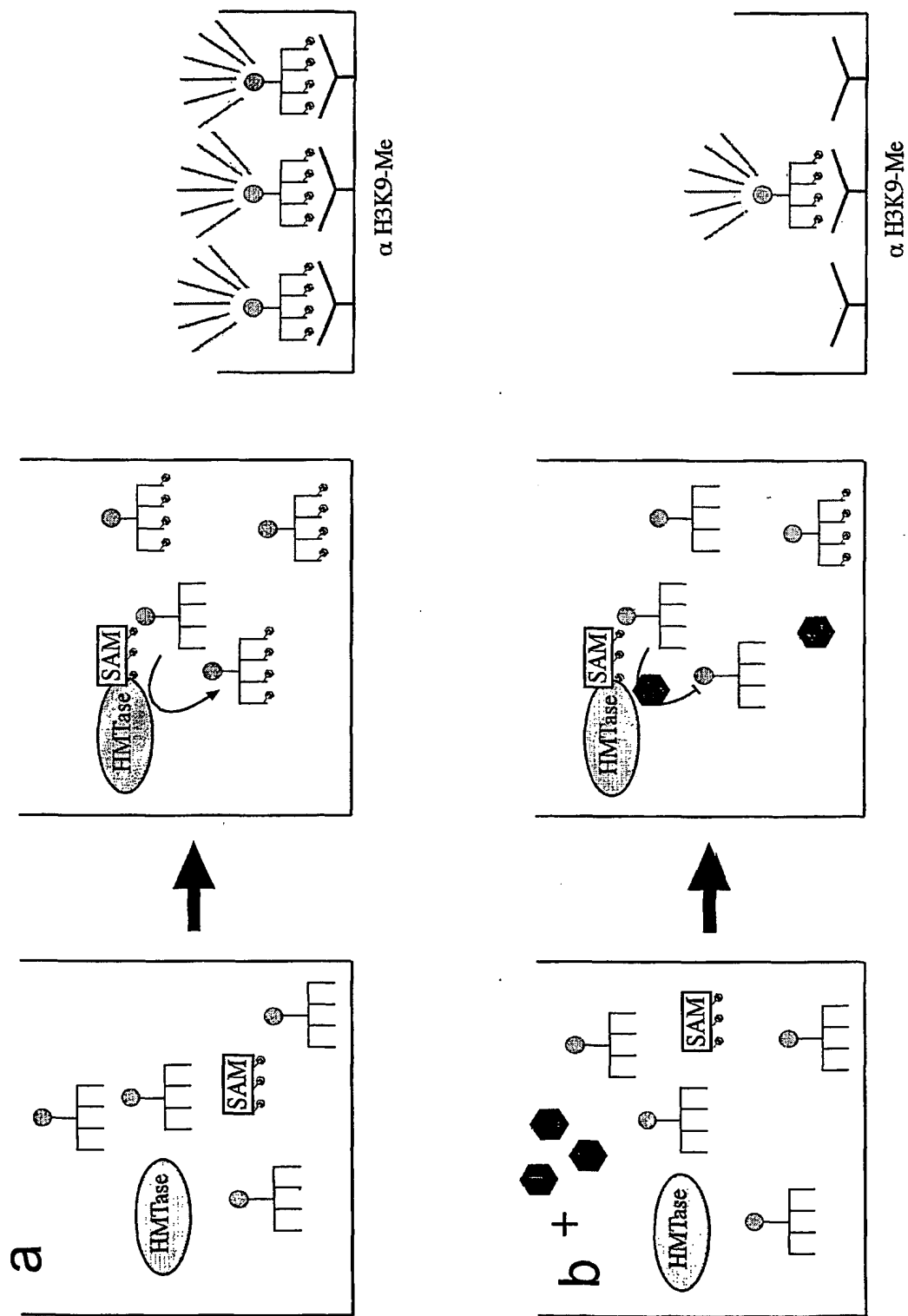


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32/33
Fig. 16/2



33/33
Fig. 17



SEQUENCE LISTING

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Ile Gly Ile Thr Lys Arg Asn Leu Asn Asn Tyr Glu Val Glu Tyr Leu	
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Cys Asp Tyr Lys Val Ala Lys Gly Val Glu Tyr Tyr Leu Val Lys Trp	
125 130 135	
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Lys Gly Trp Pro Asp Ser Thr Asn Thr Trp Glu Pro Leu Arg Asn Leu	
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 Lys Ala Trp Cys Val Pro Cys Leu Val Ser Leu Asp Thr Leu Gln Glu
 85 90 95
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 Tyr Ile Val Gln Lys Ala Lys Gln Arg Ile Ala Leu Gln Arg Trp Gln
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 Glu Tyr Arg Pro Ala Pro Gly Ile Ser Ile Asn Ser Glu Ala Thr Phe
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/06485

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/48 G01N33/68 C07K7/02 C07K16/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, EMBL, PAJ, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WALLWORK J C ET AL: "PROPERTIES OF SOLUBLE RAT BRAIN HISTONE LYSINE METHYLTRANSFERASE" MONOCLONAL ANTIBODIES IN CANCER, CLIFTON, NJ, US, vol. 252, no. 17, 10 September 1977 (1977-09-10), pages 5977-5980, XP000974350 abstract page 5978, left-hand column, line 28 - line 42</p> <p style="text-align: center;">--- -/--</p>	1,7,8,10



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

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Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

23 October 2001

Date of mailing of the international search report

07/11/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax (+31-70) 340-3016

Authorized officer

Tuyman, A

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/06485

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>VENKATESAN M ET AL: "PARTIAL PURIFICATION AND CHARACTERIZATION OF A PROTEIN LYSINE METHYL TRANSFERASE EC-2.1.1.43 FROM PLASMODIA OF PHYSARUM-POLYCEPHALUM" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 18, no. 24, 1979, pages 5365-5371, XP002154903 ISSN: 0006-2960 abstract</p>	1,7,8,10
A	<p>STRAHL BRIAN D ET AL: "Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 96, no. 26, 21 December 1999 (1999-12-21), pages 14967-14972, XP002154904 ISSN: 0027-8424 cited in the application page 14967, left-hand column, line 27 -right-hand column, line 1 page 14967, right-hand column, line 32 -page 14968, left-hand column, line 21</p>	1,2,5-8, 10
A	<p>AAGAARD LOUISE ET AL: "Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 18, no. 7, 1 April 1999 (1999-04-01), pages 1923-1938, XP002154905 ISSN: 0261-4189 cited in the application abstract page 1925; figure 1</p>	1-3,5-8, 10
A	<p>DATABASE EMBL 'Online! Ac No. : AF149204, O'CARROLL D ET AL.: "Suv maps to chromosome 2" XP002155318 the whole document</p>	1,4

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/06485

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	REA STEPHEN ET AL: "Regulation of chromatin structure by site-specific histone H3 methyltransferases" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 406, no. 6796, August 2000 (2000-08), pages 593-599, XP002154907 ISSN: 0028-0836 cited in the application abstract page 596, left-hand column, line 9 -right-hand column, line 22 page 598, right-hand column, line 42 -page 599, left-hand column, line 6	1-3,5-8, 10
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X	HOLT VON C ET AL: "ISOLATION AND CHARACTERIZATION OF HISTONES" METHODS IN ENZYMOLOGY, ACADEMIC PRESS INC, SAN DIEGO, CA, US, vol. 170, 1989, pages 431-523, XP000972597 ISSN: 0076-6879 page 516	13
A	MACFARLEN J O ET AL: "COLORIMETRIC ASSAY FOR GUAIACOL O METHYL TRANSFERASE" JOURNAL OF PHARMACEUTICAL SCIENCES, AMERICAN PHARMACEUTICAL ASSOCIATION. WASHINGTON, US, vol. 65, no. 11, 1976, pages 1689-1690, XP002156114 ISSN: 0022-3549 the whole document	1,7-9
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P,A	---	14

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/06485

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NAKAYAMA JUN-ICHI ET AL: "Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly." SCIENCE (WASHINGTON D C), vol. 292, no. 5514, 2001, pages 110-113, XP002177964 ISSN: 0036-8075 cited in the application	1,2, 5-11,13
P,A P,X	page 111, middle column, line 7 - line 24 -& NAKAYAMA JUN-ICHI ET AL.: SCIENCE-SUPPLEMENTAL DATA, 'Online!' 6 April 2001 (2001-04-06), pages 1-3, XP002177965 Retrieved from the Internet: <URL:www.sciencemag.org/cgi/content/full/1060118/DC1> 'retrieved on 2001-09-20! the whole document	12,14 1,2, 5-11,13
P,A	-----	12,14

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 01 06485

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 15-17

Present claims 15-17 relate to a compound defined by reference to a desirable characteristic or property, namely being identifiable in a method defined in any one of the claims 1-12.

The claims cover all compounds having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT nor disclosure within the meaning of Article 5 PCT for any of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for these claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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